

POLYMER-FACTOR VIII MOIETY CONJUGATES

CROSS REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of priority to U.S. Provisional Patent Application Serial No. 60/450,578, filed February 26, 2003, which is incorporated herein by reference.

FIELD OF THE INVENTION

[0002] The present invention relates generally to conjugates comprising a Factor VIII moiety (i.e., a moiety having Factor VIII activity) and a polymer. In addition, the invention relates to compositions comprising the conjugates, methods for synthesizing the conjugates, and methods for treating patients.

BACKGROUND OF THE INVENTION

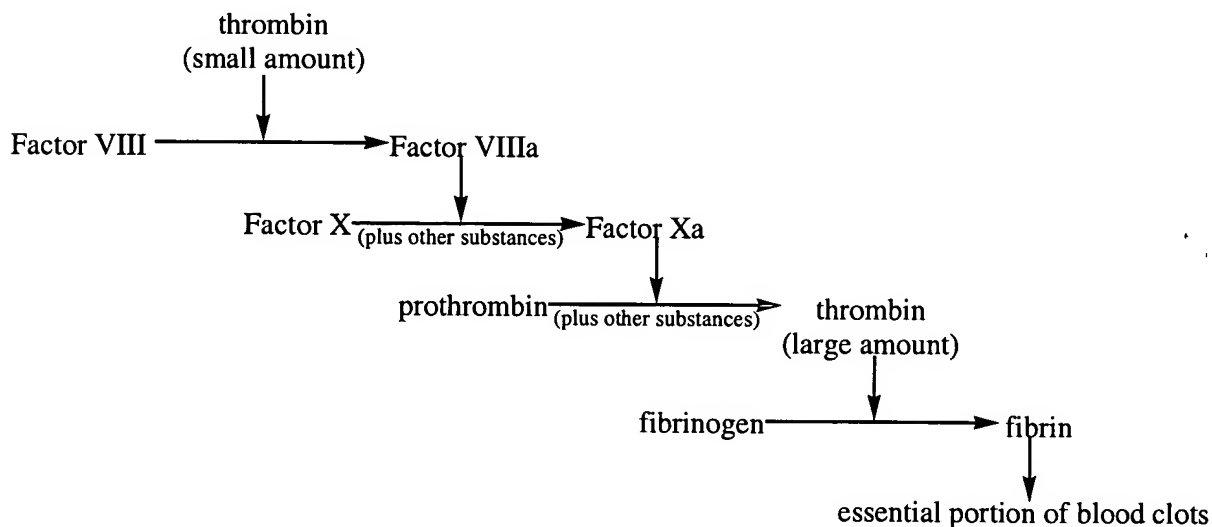
[0003] Hemostasis is the process of arresting the outflow of blood from an injured blood vessel. For mammals, as well as many other organisms, the hemostatic process is critically important for continued survival. Defects in the hemostatic process can result in, for example, the inability to effectively form blood clots that serve to stop the loss of blood following vascular injury. In humans, individuals who suffer from an inability to form blood clots are called hemophiliacs. Of particular concern for hemophiliacs is the life-threatening risk that once started, bleeding will never cease.

[0004] Generally, hemophiliacs lack the ability to produce effective amounts of one or more substances required to form blood clots. For example, hemophiliacs who suffer from hemophilia A (also called "classic hemophilia") have an inability to produce effective levels of Factor VIII (also known as "antihemophilia factor A," "antihemophilic globulin," and "AHG"). Factor VIII is a key component of one of several "cascades" of reactions that result in the formation of blood clots. Critical for the cascade of reactions referred to as the "intrinsic

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pathway," Factor VIII ultimately influences the conversion of fibrinogen into the major component of blood clots, fibrin.

[0005] Although the intrinsic pathway of blood clot formation is relatively complex, the role of Factor VIII can be described briefly. In the presence of relatively small amounts of thrombin (released, for example, by the cells of ruptured tissues), Factor VIII is converted into its activated form known as Factor VIIIa. Factor VIIIa (along with other substances), in turn, activates another factor, Factor X into Factor Xa. Thereafter, Factor Xa (along with other substances) converts prothrombin into thrombin, with the result that a relatively large amount of thrombin is produced over time. Relatively large amounts of thrombin effectively convert fibrinogen into fibrin. Fibrin, in turn, forms the matrix or lattice responsible for the formation of blood clots. Factor VIII's role in the intrinsic pathway of blood clotting is shown schematically below.



[0006] Affecting one or two males for every 10,000 live births in all populations, hemophilia A can result from any one of a variety of mutations of the Factor VIII gene, which is located on the X-chromosome. Depending on the particular mutation, hemophilia A can manifest itself as severe, moderate or mild. Individuals suffering from the severest forms of hemophilia A entirely lack the ability to express active forms of Factor VIII. Clinically, individuals affected with hemophilia A suffer from muscle hemorrhage, joint hemorrhage, easy bruising, and prolonged bleeding from wounds. The mean age of individuals suffering from

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hemophilia A without treatment is twenty. Current treatment of hemophilia A involves the infusion of exogenous Factor VIII concentrate collected from human plasma or prepared via recombinant DNA techniques. Because these treatments serve only to supplement the lack of effective levels of Factor VIII, individuals suffering from Factor VIII require regular injections of Factor VIII concentrate throughout their lives.

[0007] Several commercial forms of Factor VIII concentrates are available to provide replacement therapy for patients suffering from hemophilia A. For example, blood-derived Factor VIII concentrate products sold under the Hemofil[®] M (Baxter, Deerfield, IL), Koate[®]-DVI (Bayer, Research Triangle Park, NC), Monarc-M[™] (American Red Cross, Washington, D.C.), and Monoclote-P[®] (Aventis, Bridgewater, NJ) brands. With respect to recombinantly prepared Factor VIII concentrates, commercial products are provided under the Helixate[®] FS (Aventis, Bridgewater, NJ), Kogenate FS[®] (Bayer, Research Triangle Park, NC), Recombinate[®] (Baxter, Deerfield, IL), Advate[®] (Baxter, Deerfield, IL), and ReFacto[®] (Wyeth/Genetics Institute, Cambridge, MA) brands.

[0008] Generally, recombinant sources of Factor VIII concentrates are favored over blood-derived sources since the latter involves the risk of transmitting viruses and/or other diseases associated with blood donation. While recombinant-based formulations avoid these drawbacks, the processing of recombinant-based products often requires the presence of certain proteins such as albumin, which are inevitably present in the final formulation administered to the patient. Often, patients who receive such formulations develop allergic reactions to these foreign proteins. In any event, both blood-derived and recombinant-based products suffer from the disadvantage of repeated administration.

[0009] PEGylation, or the attachment of a poly(ethylene glycol) derivative to a protein, has been described as a means to reduce immunogenicity as well as a means to prolong a protein's *in vivo* half-life. With respect to Factor VIII, however, previous experiences with forming protein-polymer conjugates has proven to be of little predictive value vis-à-vis polymer coupling to Factor VIII. See U.S. Patent No. 4,970,300.

[0010] Notwithstanding these difficulties, attempts of preparing satisfactory compositions of conjugates of certain polymers to Factor VIII have been described. For example, previously

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referenced U.S. Patent No. 4,970,300 describes the PEGylation of Factor VIII using a specific poly(ethylene glycol) derivative having a molecular weight within the range of about 500 to 5,000. In addition, U.S. Patent No. 6,048,720 describes efficient protection against degradation in an *in vitro* environment when four to five monomethoxy poly(ethylene glycol) strands are conjugated to Factor VIII.

[0011] None of these described conjugates, however, has proven to satisfactorily address the problems associated with current Factor VIII-based therapies. For example, conjugates comprised of relatively small polymers (*e.g.*, of about 5,000 Daltons or less) may not suitably provide extended *in vivo* half-life and/or sufficiently reduced immune response. In addition, conjugates having many individual polymers attached to Factor VIII are more likely to have reduced activity as a result of the polymer(s) blocking sites necessary for activity.

[0012] Thus, there remains a need in the art to provide additional conjugates between water-soluble polymers and moieties having Factor VIII activity. The present invention is therefore directed to such conjugates as well as compositions comprising the conjugates and related methods as described herein, which are believed to be new and completely unsuggested by the art.

SUMMARY OF THE INVENTION

[0013] Accordingly, it is a primary object of this invention to provide a composition comprising a plurality of conjugates, preferably although not necessarily, each having one to three water-soluble polymers covalently attached to a Factor VIII moiety, wherein each water-soluble polymer preferably has a nominal average molecular weight in the range of greater than 5,000 Daltons to about 100,000 Daltons.

[0014] It is another object of the invention to provide such a conjugate wherein each of the water-soluble polymers is a poly(alkylene oxide).

[0015] It is an additional object of the invention to provide such a conjugate wherein each of the water-soluble polymers is a poly(ethylene glycol).

[0016] It is a further object of the invention to provide a conjugate comprising a plurality of monoPEGylated Factor VIII moiety conjugates.

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[0017] It is still a further object of the invention to provide a method for preparing polymer conjugates comprising the steps of contacting one or more activated, water-soluble polymers to a Factor VIII moiety under conditions sufficient to result in a plurality of conjugates, preferably although not necessarily, each having one to three water-soluble polymers covalently attached to a Factor VIII moiety, wherein the water-soluble polymer preferably has a nominal average molecular weight in the range of greater than 5,000 Daltons to about 150,000 Daltons.

[0018] It is an additional object of the invention to provide a method for treating a patient in need of Factor VIII therapy, comprising the step of administering to the patient a composition as described herein, wherein the composition contains a therapeutically effective amount of one or more of the conjugates.

[0019] It is still yet an additional object of the invention to provide a method for preparing a water-soluble polymer-Factor VIII moiety conjugate comprising the step of contacting, under conjugation conditions, a Factor VIII moiety with a polymeric reagent.

[0020] Additional objects, advantages and novel features of the invention will be set forth in the description that follows, and in part, will become apparent to those skilled in the art upon reading the following, or may be learned by practice of the invention.

[0021] In one embodiment then, a composition is provided comprising a plurality of conjugates, preferably although not necessarily, each having one to three-water soluble polymers covalently attached to a Factor VIII moiety, wherein each water-soluble polymer preferably has a nominal average molecular weight in the range of greater than 5,000 Daltons to about 150,000 Daltons. Although any Factor VIII moiety can be used, it is preferred that the compositions comprise Factor VIII *per se* (as described in, for example, U.S. Patent No. 4,757,006), Factor VIIIa (*i.e.*, the activated form of Factor VIII produced when Factor VIII is placed in contact with relatively small amounts of thrombin), Factor VIII:vWF (*i.e.*, Factor VIII bound to von Willebrand Factor), and/or truncated versions of Factor VIII such as B-domain deleted Factor VIII (as described in, for example, U.S. Patent No. 4,868,112).

[0022] The polymer(s) can be any water-soluble polymer and the invention is not limited in this regard. It is preferred, however, that each polymer present in the conjugate is selected from the group consisting of poly(alkylene oxide), poly(vinyl pyrrolidone), poly(vinyl alcohol),

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polyoxazoline, poly(acryloylmorpholine), and combinations thereof. It is particularly, preferred, however, that a poly(alkylene oxide) such as a poly(ethylene glycol) derivative is used as the polymer in the present invention.

[0023] The conjugates described herein advantageously reduce immunogenicity, a problem encountered by many hemophiliacs treated with exogenous sources of Factor VIII. In addition, the present conjugates require decreased frequency of dosing compared to Factor VIII compositions lacking conjugates. By reducing the frequency of dosing, the conjugates advantageously decrease the number of painful injections hemophiliacs must endure in order to provide sustained levels of an agent having Factor VIII activity.

[0024] In another embodiment, a method for preparing a conjugate is provided. The method comprises the step of contacting one or more activated, water-soluble polymers (*i.e.*, a polymeric reagent) preferably having a nominal average molecular weight in the range of greater than 5,000 Daltons to about 150,000 Daltons to a Factor VIII moiety. Activation of the water-soluble polymer can be accomplished under any art-known method so long as the resulting polymer, under the proper conditions of pH, temperature, and so forth, will form a covalent bond such that the Factor VIII moiety is covalently attached to the polymer. Contacting of the one or more activated, water-soluble polymers to the Factor VIII moiety is carried out under those conditions required for the activated, water-soluble polymer to form a covalent attachment at the desired site in the moiety. The method results in a plurality of conjugates, preferably although not necessarily, each having one to three water-soluble polymers covalently attached to the Factor VIII moiety. In some instances, the conjugate can comprise a single polymer attached to two, three, four, five, six, seven, eight, or more Factor VIII moieties. Optionally, the resulting composition can be further processed in order to remove undesired species such as, for example, conjugates having an undesired number of polymers. In order to remove such undesired species, purification techniques such as size-exclusion chromatography can be used.

[0025] In still another embodiment of the invention, compositions are provided comprising a conjugate of the invention in combination with a pharmaceutically acceptable excipient. The compositions encompass all types of formulations and in particular those that are suited for injection such as powders that can be reconstituted, as well as liquids (*e.g.*, suspensions and solutions).

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[0026] In an additional embodiment of the invention, a method of administering the conjugate is provided. The method of administering comprises the step of administering to the patient a composition as described herein, wherein the composition contains a therapeutically effective amount of the conjugate. Typically, the step of administering the conjugate-containing composition is effected by injection (*e.g.*, intramuscular injection, intravenous injection, subcutaneous injection, and so forth).

BRIEF DESCRIPTION OF THE FIGURES

[0027] FIG. 1 is a SEC plot corresponding to the reaction mixture formed upon pegylation of B-Domain deleted Factor VIII with mPEG-SPA, 30K, as described in Example 6.

[0028] FIG. 2 is a SEC plot corresponding to purified B-Domain Deleted Factor VIII-PEG conjugate prepared by conjugating the protein to mPEG-SPA, 30K, as described in Example 6.

[0029] FIG. 3 is SEC plot corresponding to the reaction mixture formed upon PEGylation of B-Domain deleted Factor VIII with mPEG-MAL, 20K, as described in Example 7. As can be seen, the yield of monoPEGylated product was approximately 33%.

[0030] FIG. 4 is a SEC plot corresponding to purified B-Domain Deleted Factor VIII-PEG conjugate prepared by conjugating the protein to mPEG-MAL, 20K, as described in Example 7. The purified conjugate was ~94% PEG monomer.

[0031] FIG. 5 is a SEC plot corresponding to the reaction mixture formed upon PEGylation of B-Domain Deleted Factor VIII with mPEG-SMB, 30K, as described in Example 8. The yield of monoPEGylated protein (1-mer) was approximately 41%.

DETAILED DESCRIPTION OF THE INVENTION

[0032] Before describing the present invention in detail, it is to be understood that this invention is not limited to the particular polymers, synthetic techniques, Factor VIII moieties, and the like, as such may vary.

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[0033] It must be noted that, as used in this specification and the intended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polymer" includes a single polymer as well as two or more of the same or different polymers, reference to a "an optional excipient" refers to a single optional excipient as well as two or more of the same or different optional excipients, and the like.

[0034] In describing and claiming the present invention, the following terminology will be used in accordance with the definitions described below.

[0035] "PEG," "polyethylene glycol" and "poly(ethylene glycol)" as used herein, are interchangeable and meant to encompass any water-soluble poly(ethylene oxide). Typically, PEGs for use in accordance with the invention comprise the following structure $-(\text{OCH}_2\text{CH}_2)_n-$ where (n) is 2 to 4000. As used herein, PEG also includes $-\text{CH}_2\text{CH}_2-\text{O}(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2-$ and $-(\text{OCH}_2\text{CH}_2)_n\text{O}-$, depending upon whether or not the terminal oxygens have been displaced. Throughout the specification and claims, it should be remembered that the term "PEG" includes structures having various terminal or "end capping" groups and so forth. The term "PEG" also means a polymer that contains a majority, that is to say, greater than 50%, of $-\text{OCH}_2\text{CH}_2-$ repeating subunits. With respect to specific forms, the PEG can take any number of a variety of molecular weights, as well as structures or geometries such as "branched," "linear," "forked," "multifunctional," and the like, to be described in greater detail below.

[0036] The terms "end-capped" and "terminally capped" are interchangeably used herein to refer to a terminal or endpoint of a polymer having an end-capping moiety. Typically, although not necessarily, the end-capping moiety comprises a hydroxy or C_{1-20} alkoxy group, more preferably a C_{1-10} alkoxy group, and still more preferably a C_{1-5} alkoxy group. Thus, examples of end-capping moieties include alkoxy (e.g., methoxy, ethoxy and benzyloxy), as well as aryl, heteroaryl, cyclo, heterocyclo, and the like. It must be remembered that the end-capping moiety may include one or more atoms of the terminal monomer in the polymer [e.g., the end-capping moiety "methoxy" in $\text{CH}_3(\text{OCH}_2\text{CH}_2)_n-$]. In addition, saturated, unsaturated, substituted and unsubstituted forms of each of the foregoing are envisioned. Moreover, the end-capping group can also be a silane. The end-capping group can also advantageously comprise a detectable label. When the polymer has an end-capping group comprising a

detectable label, the amount or location of the polymer and/or the moiety (*e.g.*, active agent) to which the polymer is coupled can be determined by using a suitable detector. Such labels include, without limitation, fluorescers, chemiluminescers, moieties used in enzyme labeling, colorimetric (*e.g.*, dyes), metal ions, radioactive moieties, and the like. Suitable detectors include photometers, films, spectrometers, and the like. The end-capping group can also advantageously comprise a phospholipid. When the polymer has an end-capping group comprising a phospholipid, unique properties are imparted to the polymer and the resulting conjugate. Exemplary phospholipids include, without limitation, those selected from the class of phospholipids called phosphatidylcholines. Specific phospholipids include, without limitation, those selected from the group consisting of dilauroylphosphatidylcholine, dioleoylphosphatidylcholine, dipalmitoylphosphatidylcholine, disteoylphosphatidylcholine, behenoylphosphatidylcholine, arachidoylphosphatidylcholine, and lecithin.

[0037] "Non-naturally occurring" with respect to a polymer as described herein, means a polymer that in its entirety is not found in nature. A non-naturally occurring polymer of the invention may, however, contain one or more monomers or segments of monomers that are naturally occurring, so long as the overall polymer structure is not found in nature.

[0038] The term "water soluble" as in a "water-soluble polymer" polymer is any polymer that is soluble in water at room temperature. Typically, a water-soluble polymer will transmit at least about 75%, more preferably at least about 95%, of light transmitted by the same solution after filtering. On a weight basis, a water-soluble polymer will preferably be at least about 35% (by weight) soluble in water, more preferably at least about 50% (by weight) soluble in water, still more preferably about 70% (by weight) soluble in water, and still more preferably about 85% (by weight) soluble in water. It is most preferred, however, that the water-soluble polymer is about 95% (by weight) soluble in water or completely soluble in water.

[0039] "Nominal average molecular weight" in the context of a water-soluble, non-naturally occurring polymer such as PEG, refers to the mass average molecular weight of the polymer, typically determined by size-exclusion chromatography, MALDI (matrix assisted laser desorption/ionization), light scattering techniques, or intrinsic velocity determination in 1,2,4-trichlorobenzene. The polymers are typically polydisperse, possessing low polydispersity values of preferably less than about 1.2, more preferably less than about 1.15, still more

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preferably less than about 1.10, yet still more preferably less than about 1.05, and most preferably less than about 1.03.

[0040] The term "active" or "activated" when used in conjunction with a particular functional group, refers to a reactive functional group that reacts readily with an electrophile or a nucleophile on another molecule. This is in contrast to those groups that require strong catalysts or highly impractical reaction conditions in order to react (*i.e.*, a "non-reactive" or "inert" group).

[0041] As used herein, the term "functional group" or any synonym thereof is meant to encompass protected forms thereof as well as unprotected forms.

[0042] The terms "linkage" or "linker" are used herein to refer to an atom or a collection of atoms optionally used to link interconnecting moieties such as a terminus of a polymer segment and a Factor VIII moiety or an electrophile or nucleophile of a Factor VIII moiety. The linker of the invention may be hydrolytically stable or may include a physiologically hydrolyzable or enzymatically degradable linkage.

[0043] "Alkyl" refers to a hydrocarbon chain, typically ranging from about 1 to 15 atoms in length. Such hydrocarbon chains are preferably but not necessarily saturated and may be branched or straight chain, although typically straight chain is preferred. Exemplary alkyl groups include methyl, ethyl, propyl, butyl, pentyl, 1-methylbutyl, 1-ethylpropyl, 3-methylpentyl, and the like. As used herein, "alkyl" includes cycloalkyl as well as cycloalkylene-containing alkyl.

[0044] "Lower alkyl" refers to an alkyl group containing from 1 to 6 carbon atoms, and may be straight chain or branched, as exemplified by methyl, ethyl, *n*-butyl, *i*-butyl, and *t*-butyl.

[0045] "Cycloalkyl" refers to a saturated or unsaturated cyclic hydrocarbon chain, including bridged, fused, or spiro cyclic compounds, preferably made up of 3 to about 12 carbon atoms, more preferably 3 to about 8 carbon atoms. "Cycloalkylene" refers to a cycloalkyl group that is inserted into an alkyl chain by bonding of the chain at any two carbons in the cyclic ring system.

[0046] "Alkoxy" refers to an -O-R group, wherein R is alkyl or substituted alkyl, preferably C₁₋₆ alkyl (*e.g.*, methoxy, ethoxy, propyloxy, and so forth).

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[0047] The term "substituted" as in, for example, "substituted alkyl," refers to a moiety (*e.g.*, an alkyl group) substituted with one or more noninterfering substituents, such as, but not limited to: alkyl, C₃₋₈ cycloalkyl, *e.g.*, cyclopropyl, cyclobutyl, and the like; halo, *e.g.*, fluoro, chloro, bromo, and iodo; cyano; alkoxy, lower phenyl; substituted phenyl; and the like.

"Substituted aryl" is aryl having one or more noninterfering groups as a substituent. For substitutions on a phenyl ring, the substituents may be in any orientation (*i.e.*, ortho, meta, or para).

[0048] "Noninterfering substituents" are those groups that, when present in a molecule, are typically nonreactive with other functional groups contained within the molecule.

[0049] "Aryl" means one or more aromatic rings, each of 5 or 6 core carbon atoms. Aryl includes multiple aryl rings that may be fused, as in naphthyl or unfused, as in biphenyl. Aryl rings may also be fused or unfused with one or more cyclic hydrocarbon, heteroaryl, or heterocyclic rings. As used herein, "aryl" includes heteroaryl.

[0050] "Heteroaryl" is an aryl group containing from one to four heteroatoms, preferably sulfur, oxygen, or nitrogen, or a combination thereof. Heteroaryl rings may also be fused with one or more cyclic hydrocarbon, heterocyclic, aryl, or heteroaryl rings.

[0051] "Heterocycle" or "heterocyclic" means one or more rings of 5-12 atoms, preferably 5-7 atoms, with or without unsaturation or aromatic character and having at least one ring atom that is not a carbon. Preferred heteroatoms include sulfur, oxygen, and nitrogen.

[0052] "Substituted heteroaryl" is heteroaryl having one or more noninterfering groups as substituents.

[0053] "Substituted heterocycle" is a heterocycle having one or more side chains formed from noninterfering substituents.

[0054] "Electrophile" and "electrophilic group" refer to an ion or atom or collection of atoms, that may be ionic, having an electrophilic center, *i.e.*, a center that is electron seeking, capable of reacting with a nucleophile.

[0055] "Nucleophile" and "nucleophilic group" refers to an ion or atom or collection of atoms that may be ionic having a nucleophilic center, *i.e.*, a center that is seeking an electrophilic center or with an electrophile.

[0056] A "physiologically cleavable" or "hydrolysable" or "degradable" bond is a bond that reacts with water (*i.e.*, is hydrolyzed) under physiological conditions. Preferred are bonds that have a hydrolysis half-life at pH 8, 25 °C of less than about 30 minutes. The tendency of a bond to hydrolyze in water will depend not only on the general type of linkage connecting two central atoms but also on the substituents attached to these central atoms. Appropriate hydrolytically unstable or weak linkages include but are not limited to carboxylate ester, phosphate ester, anhydrides, acetals, ketals, acyloxyalkyl ether, imines, orthoesters, peptides and oligonucleotides.

[0057] An "enzymatically degradable linkage" means a linkage that is subject to degradation by one or more enzymes.

[0058] A "hydrolytically stable" linkage or bond refers to a chemical bond, typically a covalent bond, that is substantially stable in water, that is to say, does not undergo hydrolysis under physiological conditions to any appreciable extent over an extended period of time. Examples of hydrolytically stable linkages include, but are not limited to, the following: carbon-carbon bonds (*e.g.*, in aliphatic chains), ethers, amides, urethanes, and the like. Generally, a hydrolytically stable linkage is one that exhibits a rate of hydrolysis of less than about 1-2% per day under physiological conditions. Hydrolysis rates of representative chemical bonds can be found in most standard chemistry textbooks.

[0059] "Pharmaceutically acceptable excipient or carrier" refers to an excipient that may optionally be included in the compositions of the invention and that causes no significant adverse toxicological effects to the patient. "Pharmacologically effective amount," "physiologically effective amount," and "therapeutically effective amount" are used interchangeably herein to mean the amount of a polymer-Factor VIII moiety conjugate that is needed to provide a desired level of the conjugate (or corresponding unconjugated Factor VIII moiety) in the bloodstream or in the target tissue. The precise amount will depend upon numerous factors, *e.g.*, the particular Factor VIII moiety, the components and physical characteristics of the therapeutic composition, intended

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patient population, individual patient considerations, and the like, and can readily be determined by one skilled in the art, based upon the information provided herein.

[0060] "Multi-functional" means a polymer having 3 or more functional groups contained therein, where the functional groups may be the same or different. Multi-functional polymeric reagents of the invention will typically contain from about 3-100 functional groups, or from 3-50 functional groups, or from 3-25 functional groups, or from 3-15 functional groups, or from 3 to 10 functional groups, or will contain 3, 4, 5, 6, 7, 8, 9 or 10 functional groups within the polymer backbone.

[0061] The term "Factor VIII moiety," as used herein, refers to a moiety having Factor VIII activity. The Factor VIII moiety will also have at least one electrophilic group or nucleophilic group suitable for reaction with a polymeric reagent. Typically, although not necessarily, the Factor VIII moiety is a protein. In addition, the term "Factor VIII moiety" encompasses both the Factor VIII moiety prior to conjugation as well as the Factor VIII moiety residue following conjugation. As will be explained in further detail below, one of ordinary skill in the art can determine whether any given moiety has Factor VIII activity.

[0062] The term "patient," refers to a living organism suffering from or prone to a condition that can be prevented or treated by administration of an active agent (*e.g.*, conjugate), and includes both humans and animals.

[0063] "Optional" or "optionally" means that the subsequently described circumstance may or may not occur, so that the description includes instances where the circumstance occurs and instances where it does not.

[0064] Amino acid residues in peptides are abbreviated as follows: Phenylalanine is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Valine is Val or V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; and Glycine is Gly or G.

[0065] Turning to a first embodiment of the invention then, a composition is provided comprising a plurality of conjugates, preferably although not necessarily, each having one to

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three water-soluble polymers covalently attached to a Factor VIII moiety, wherein each of the water-soluble polymers preferably has a nominal average molecular weight in the range of greater than 5,000 Daltons to about 150,000 Daltons.

[0066] Native Factor VIII is a 2,351 amino acid, single chain glycoprotein that is structurally organized as A1-A2-B-A3-C1-C2. The expressed 2,351 amino acid sequence is provided as SEQ. ID. NO: 1. When the expressed polypeptide is translocated into the lumen of the endoplasmic reticulum, however, a 19-amino acid signal sequence is cleaved, resulting in a second sequence. This second sequence, herein provided as SEQ. ID. NO: 2, lacks the leading 19 amino acids is conventionally used by researchers to assign a numeric location (*e.g.*, Arg³⁷²) to a given amino acid residue of Factor VIII. Thus, unless specifically noted, all assignments of a numeric location of an amino acid residue as provided herein are based on SEQ. ID. NO: 2.

[0067] In the presence of relatively small amounts of thrombin, Factor VIII is cleaved by thrombin at Arg³⁷², Arg⁷⁴⁰, and Arg¹⁶⁸⁹ to produce Factor VIIIa. Factor VIIIa is a heterotrimer comprised of the A1 subunit bound (via a copper ion) to the thrombin-cleaved light chain A3-C1-C2 and the free A2 subunit bound via ionic interactions to A1. It will be appreciated that a Factor VIII moiety is not limited to merely "active" forms of Factor VIII (*e.g.*, Factor VIIIa) and that the term "Factor VIII moiety" encompasses "precursor" forms as well as other substances that having a similar procoagulant effect .

[0068] For any given moiety, it is possible to determine whether that moiety has Factor VIII activity. For example, several animal lines have been intentionally bred with the genetic mutation for hemophilia such that an animal produced from such a line has very low and insufficient levels of Factor VIII. Such lines are available from a variety of sources such as, without limitation, the Division of Laboratories and Research, New York Department of Public Health, Albany, NY and the Department of Pathology, University of North Carolina, Chapel Hill, NC. Both of these sources, for example, provide canines suffering from canine hemophilia A. In order to test the Factor VIII activity of any given moiety in question, the moiety is injected into the diseased animal, a small cut made and bleeding time compared to a healthy control. Another method useful for determining Factor VIII activity is to determine cofactor and procoagulant activity. See, for example, Mertens *et al.* (1993) *Brit. J. Haematol.* 85:133-42. Other methods known to those of ordinary skill in the art can also be used to determine whether a given moiety

has Factor VIII activity. Such methods are useful for determining the Factor VIII activity of both the moiety itself (and therefore can be used as a "Factor VIII moiety) as well as the corresponding polymer-moiety conjugate.

[0069] Nonlimiting examples of Factor VIII moieties include the following: Factor VIII; Factor VIIIa; Factor VIII:C; Factor VIII:vWF; B-domain deleted Factor VIII (and other truncated versions of Factor VIII); hybrid proteins, such as those described in U.S. Patent No. 6,158,888; glycosylated proteins having Factor VIII activity, such as those described in U.S. Patent Application Publication No. US2003/0077752; and peptide mimetics having Factor VIII activity. Preferred truncated Factor VIII versions (encompassed by the term "B-domain deleted Factor VIII) corresponds to a protein having the amino acid sequence of human Factor VIII (SEQ. ID. NO. 1) having a deletion corresponding to at least 581 amino acids within the region between Arg⁷⁵⁹ and Ser¹⁷⁰⁹, more preferably wherein the deletion corresponds to one of the region between Pro¹⁰⁰⁰ and Asp¹⁵⁸², the region between Thr⁷⁷⁸ and Pro¹⁶⁵⁹, and the region between Thr⁷⁷⁸ and Glu¹⁶⁹⁴. Biologically active fragments, deletion variants, substitution variants or addition variants of any of the foregoing that maintain at least some degree of Factor VIII activity can also serve as a Factor VIII moiety.

[0070] The moiety having Factor VIII activity can advantageously be modified to include one or more amino acid residues such as, for example, lysine, cysteine and/or arginine, in order to provide facile attachment of the polymer to an atom within the side chain of the amino acid. Techniques for adding amino acid residues are well known to those of ordinary skill in the art. Reference is made to J. March, *Advanced Organic Chemistry: Reactions Mechanisms and Structure*, 4th Ed. (New York: Wiley-Interscience, 1992).

[0071] The Factor VIII moiety can be obtained from blood-derived sources. For example, Factor VIII can be fractionated from human plasma using precipitation and centrifugation techniques known to those of ordinary skill in the art. See, for example, Wickerhauser (1976) *Transfusion* 16(4):345-350 and Slichter *et al.* (1976) *Transfusion* 16(6):616-626. Factor VIII can also be isolated from human granulocytes. See Szmitkoski *et al.* (1977) *Haematologia (Budap.)* 11(1-2):177-187.

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[0072] In addition, the Factor VIII moiety can also be obtained from recombinant methods. Briefly, recombinant methods involve constructing the nucleic acid encoding the desired polypeptide or fragment, cloning the nucleic acid into an expression vector, transforming a host cell (*e.g.*, bacteria, yeast, or mammalian cell such as Chinese hamster ovary cell or baby hamster kidney cell), and expressing the nucleic acid to produce the desired polypeptide or fragment. Methods for producing and expressing recombinant polypeptides *in vitro* and in prokaryotic and eukaryotic host cells are known to those of ordinary skill in the art. See, for example, U.S. Patent No. 4,868,122.

[0073] To facilitate identification and purification of the recombinant polypeptide, nucleic acid sequences that encode for an epitope tag or other affinity binding sequence can be inserted or added in-frame with the coding sequence, thereby producing a fusion protein comprised of the desired polypeptide and a polypeptide suited for binding. Fusion proteins can be identified and purified by first running a mixture containing the fusion protein through an affinity column bearing binding moieties (*e.g.*, antibodies) directed against the epitope tag or other binding sequence in the fusion proteins, thereby binding the fusion protein within the column. Thereafter, the fusion protein can be recovered by washing the column with the appropriate solution (*e.g.*, acid) to release the bound fusion protein. The recombinant polypeptide can also be identified and purified by lysing the host cells, separating the polypeptide, *e.g.*, by size exclusion chromatography, and collecting the polypeptide. These and other methods for identifying and purifying recombinant polypeptides are known to those of ordinary skill in the art.

[0074] The compositions of the invention can comprise a plurality of conjugates, each conjugate comprised of the same Factor VIII moiety (*i.e.*, within the entire composition, only one type of Factor VIII moiety is found). In addition, the composition can comprise a plurality of conjugates wherein any given conjugate is comprised of a moiety selected from the group consisting of two or more different Factor VIII moieties (*i.e.*, within the entire composition, two or more different Factor VIII moieties are found). Optimally, however, substantially all of the plurality of conjugates in the composition (*e.g.*, 85% or more of the plurality of conjugates in the composition) are each comprised of the same Factor VIII moiety.

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[0075] Moreover, it is preferred that the composition containing the conjugates is free or substantially free from albumin. It is also preferred that the composition is free or substantially free proteins that do not have Factor VIII activity. Thus, it is preferred that the composition is 85%, more preferably 95%, and most preferably 99% free from albumin. Additionally, it is preferred that the composition is 85%, more preferably 95%, and most preferably 99% free from any protein that does not have Factor VIII activity.

[0076] As previously discussed, each conjugate comprises a Factor VIII moiety attached to a water-soluble polymer. With respect to the water-soluble polymer, the water-soluble polymer is nonpeptidic, nontoxic, non-naturally occurring and biocompatible. With respect to biocompatibility, a substance is considered biocompatible if the beneficial effects associated with use of the substance alone or with another substance (*e.g.*, active agent such a Factor VIII moiety) in connection with living tissues (*e.g.*, administration to a patient) outweighs any deleterious effects as evaluated by a clinician, *e.g.*, a physician. With respect to non-immunogenicity, a substance is considered nonimmunogenic if the intended use of the substance *in vivo* does not produce an undesired immune response (*e.g.*, the formation of antibodies) or, if an immune response is produced, that such a response is not deemed clinically significant or important as evaluated by a clinician. It is particularly preferred that the water-soluble polymer is biocompatible and nonimmunogenic.

[0077] Further the polymer is typically characterized as having from 2 to about 300 termini. Examples of such polymers include, but are not limited to, poly(alkylene glycols) such as polyethylene glycol (PEG), poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(α -hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazoline, poly(N-acryloylmorpholine), and combinations of any of the foregoing.

[0078] The polymer is not limited in a particular structure and can be linear (*e.g.*, alkoxy PEG or bifunctional PEG), branched or multi-armed (*e.g.*, forked PEG or PEG attached to a polyol core), dendritic, or with degradable linkages. Moreover, the internal structure of the polymer can be organized in any of number of different patterns and can be selected from the

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group consisting of homopolymer, alternating copolymer, random copolymer, block copolymer, alternating tripolymer, random tripolymer, and block tripolymer.

[0079] Typically, PEG and other water-soluble polymers are activated with a suitable activating group appropriate for coupling to a desired site on the Factor VIII moiety. An activated polymeric reagent will possess a reactive group for reaction with the Factor VIII moiety. Representative polymeric reagents and methods for conjugating these polymers to an active moiety are known in the art and further described in Zalipsky, S., *et al.*, "Use of Functionalized Poly(Ethylene Glycols) for Modification of Polypeptides" in Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications, J. M. Harris, Plenus Press, New York (1992), and in Zalipsky (1995) *Advanced Drug Reviews* 16:157-182.

[0080] Typically, the nominal average molecular weight of any given polymer in the conjugate is from about 100 Daltons to about 150,000 Daltons. Exemplary ranges, however, include nominal average molecular weights in the range of greater than 5,000 Daltons to about 100,000 Daltons, in the range of from about 6,000 Daltons to about 90,000 Daltons, in the range of from about 10,000 Daltons to about 85,000 Daltons, in the range of from about 20,000 Daltons to about 85,000 Daltons, and in the range of from about 53,000 Daltons to about 85,000 Daltons. For any given water-soluble polymer, PEGs having these molecular weight ranges are preferred.

[0081] Exemplary nominal average molecular weights for the water-soluble polymer segment include about 100 Daltons, about 200 Daltons, about 300 Daltons, about 400 Daltons, about 500 Daltons, about 600 Daltons, about 700 Daltons, about 750 Daltons, about 800 Daltons, about 900 Daltons, about 1,000 Daltons, about 2,000 Daltons, about 2,200 Daltons, about 2,500 Daltons, about 3,000 Daltons, about 4,000 Daltons, about 4,400 Daltons, about 5,000 Daltons, about 6,000 Daltons, about 7,000 Daltons, about 7,500 Daltons, about 8,000 Daltons, about 9,000 Daltons, about 10,000 Daltons, about 11,000 Daltons, about 12,000 Daltons, about 13,000 Daltons, about 14,000 Daltons, about 15,000 Daltons, about 20,000 Daltons, about 22,500 Daltons, about 25,000 Daltons, about 30,000 Daltons, about 40,000 Daltons, about 50,000 Daltons, about 60,000 Daltons, and about 75,000 Daltons.

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[0082] When used as the polymer, PEGs will typically comprise a number of (OCH₂CH₂) monomers. As used throughout the description, the number of repeating units is identified by the subscript "n" in "(OCH₂CH₂)_n." Thus, the value of (n) typically falls within one or more of the following ranges: from 2 to about 2,300, from about 100 to about 2,300, from about 135 to about 2,000, from about 230 to about 1,900, from about 450 to about 1,900, and from about 1,200 to about 1,900. For any given polymer in which the molecular weight is known, it is possible to determine the number of repeating units (i.e., "n") by dividing the total molecular weight of the polymer by the molecular weight of the repeating unit.

[0083] One particularly preferred polymer for use in the invention is an end-capped polymer, that is, a polymer having at least one terminus capped with a relatively inert group, such as a lower C₁₋₆ alkoxy group. When the polymer is PEG, for example, it is preferred to use a methoxy-PEG (commonly referred to as mPEG), which is a linear form of PEG wherein one terminus of the polymer is a methoxy (-OCH₃) group, while the other terminus is a hydroxyl or other functional group that can be optionally chemically modified.

[0084] In one form useful in the present invention, free or unbound PEG is a linear polymer terminated at each end with hydroxyl groups:



wherein (m') typically ranges from zero to about 4,000.

[0085] The above polymer, alpha-, omega-dihydroxypoly(ethylene glycol), can be represented in brief form as HO-PEG-OH where it is understood that the -PEG- symbol can represent the following structural unit:



wherein (m') is as defined as above.

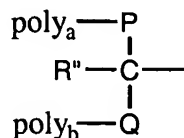
[0086] Another type of PEG useful in the present invention is methoxy-PEG-OH, or mPEG in brief, in which one terminus is the relatively inert methoxy group, while the other terminus is a hydroxyl group. The structure of mPEG is given below.



wherein (m') is as described above.

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[0087] Multi-armed or branched PEG molecules, such as those described in U.S. Patent No. 5,932,462, can also be used as the PEG polymer. For example, PEG can have the structure:



[0088] wherein:

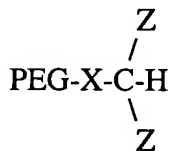
[0089] poly_a and poly_b are PEG backbones (either the same or different), such as methoxy poly(ethylene glycol);

[0090] R'' is a nonreactive moiety, such as H, methyl or a PEG backbone; and

[0091] P and Q are nonreactive linkages. In a preferred embodiment, the branched PEG polymer is methoxy poly(ethylene glycol) disubstituted lysine. Depending on the specific Factor VIII moiety used, the reactive ester functional group of the disubstituted lysine may be further modified to form a functional group suitable for reaction with the target group within the Factor VIII moiety.

[0092] These polymers may be linear, or may be in any of the above-described forms.

[0093] In addition, the PEG can comprise a forked PEG. An example of a forked PEG is represented by the following structure:

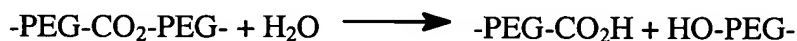


[0094] wherein: X is a spacer moiety of one or more atoms and each Z is an activated terminal group linked to CH by a chain of atoms of defined length. International Application No. PCT/US99/05333, discloses various forked PEG structures capable of use in the present invention. The chain of atoms linking the Z functional groups to the branching carbon atom serve as a tethering group and may comprise, for example, alkyl chains, ether chains, ester chains, amide chains and combinations thereof.

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[0095] The PEG polymer may comprise a pendant PEG molecule having reactive groups, such as carboxyl, covalently attached along the length of the PEG rather than at the end of the PEG chain. The pendant reactive groups can be attached to the PEG directly or through a spacer moiety, such as an alkylene group.

[0096] In addition to the above-described forms of PEG, the polymer can also be prepared with one or more weak or degradable linkages in the polymer, including any of the above described polymers. For example, PEG can be prepared with ester linkages in the polymer that are subject to hydrolysis. As shown below, this hydrolysis results in cleavage of the polymer into fragments of lower molecular weight:



[0097] Other hydrolytically degradable linkages, useful as a degradable linkage within a polymer backbone, include: carbonate linkages; imine linkages resulting, for example, from reaction of an amine and an aldehyde (see, *e.g.*, Ouchi *et al.* (1997) *Polymer Preprints* 38(1):582-3); phosphate ester linkages formed, for example, by reacting an alcohol with a phosphate group; hydrazone linkages which are typically formed by reaction of a hydrazide and an aldehyde; acetal linkages that are typically formed by reaction between an aldehyde and an alcohol; orthoester linkages that are, for example, formed by reaction between a formate and an alcohol; amide linkages formed by an amine group, *e.g.*, at an end of a polymer such as PEG, and a carboxyl group of another PEG chain; urethane linkages formed from reaction of, *e.g.*, a PEG with a terminal isocyanate group and a PEG alcohol; peptide linkages formed by an amine group, *e.g.*, at an end of a polymer such as PEG, and a carboxyl group of a peptide; and oligonucleotide linkages formed by, for example, a phosphoramidite group, *e.g.*, at the end of a polymer, and a 5' hydroxyl group of an oligonucleotide.

[0098] Such optional features of the polymer conjugate, *i.e.*, the introduction of one or more degradable linkages into the polymer chain, may provide for additional control over the final desired pharmacological properties of the conjugate upon administration. For example, a large and relatively inert conjugate (*i.e.*, having one or more high molecular weight PEG chains attached thereto, for example, one or more PEG chains having a molecular weight greater than about 10,000, wherein the conjugate possesses essentially no bioactivity) may be administered,

which is hydrolyzed to generate a bioactive conjugate possessing a portion of the original PEG chain. In this way, the properties of the conjugate can be more effectively tailored to balance the bioactivity of the conjugate over time.

[0099] Those of ordinary skill in the art will recognize that the foregoing discussion concerning substantially water-soluble polymer segments is by no means exhaustive and is merely illustrative, and that all polymeric materials having the qualities described above are contemplated. As used herein, the term "polymeric reagent" generally refers to an entire molecule, which can comprise a water-soluble polymer segment and a functional group.

[0100] As described above, a conjugate of the invention comprises a water-soluble polymer covalently attached to a Factor VIII moiety. Typically, for any given conjugate, there will be one to three water-soluble polymers covalently attached to one or more moieties having Factor VIII activity. In some instances, however, the conjugate may have 1, 2, 3, 4, 5, 6, 7, 8 or more water-soluble polymers individually attached to a Factor VIII moiety.

[0101] The particular linkage within the moiety having Factor VIII activity and the polymer depends on a number of factors. Such factors include, for example, the particular linkage chemistry employed, the particular moiety having Factor VIII activity, the available functional groups within the moiety having Factor VIII activity (either for attachment to a polymer or conversion to a suitable attachment site), the possible presence of additional reactive functional groups within the moiety having Factor VIII activity, and the like.

[0102] The conjugates of the invention can be, although not necessarily, prodrugs, meaning that the linkage between the polymer and the Factor VIII moiety is hydrolytically degradable to allow release of the parent moiety. Exemplary degradable linkages include carboxylate ester, phosphate ester, thiolester, anhydrides, acetals, ketals, acyloxyalkyl ether, imines, orthoesters, peptides and oligonucleotides. Such linkages can be readily prepared by appropriate modification of either the Factor VIII moiety (*e.g.*, the carboxyl group C terminus of the protein or a side chain hydroxyl group of an amino acid such as serine or threonine contained within the protein) and/or the polymeric reagent using coupling methods commonly employed in the art. Most preferred, however, are hydrolyzable linkages that are readily formed by reaction

of a suitably activated polymer with a non-modified functional group contained within the moiety having Factor VIII activity.

[0103] Alternatively, a hydrolytically stable linkage, such as an amide, urethane (also known as carbamate), amine, thioether (also known as sulfide), or urea (also known as carbamide) linkage can also be employed as the linkage for coupling the Factor VIII moiety. In some cases, however, it is preferred that the linkage is not a carbamate linkage and not a carbamide linkage, and furthermore, that no linkage is formed based on the reaction of a polymer derivative bearing an isocyanate or isothiocyanate species to a Factor VIII moiety. Again, a preferred hydrolytically stable linkage is an amide. An amide can be readily prepared by reaction of a carboxyl group contained within the Factor VIII moiety (*e.g.*, the terminal carboxyl of a peptidic moiety having Factor VIII activity) with an amino-terminated polymer.

[0104] The conjugates (as opposed to an unconjugated Factor VIII moiety) may or may not possess a measurable degree of Factor VIII activity. That is to say, a polymer conjugate in accordance with the invention will possess anywhere from about 0.1% to about 100% or more of the bioactivity of the unmodified parent Factor VIII moiety. Preferably, compounds possessing little or no Factor VIII activity typically contain a hydrolyzable linkage connecting the polymer to the moiety, so that regardless of the lack of activity in the conjugate, the active parent molecule (or a derivative thereof) is released upon aqueous-induced cleavage of the hydrolyzable linkage. Such activity may be determined using a suitable *in-vivo* or *in-vitro* model, depending upon the known activity of the particular moiety having Factor VIII activity employed.

[0105] For conjugates possessing a hydrolytically stable linkage that couples the moiety having Factor VIII activity to the polymer, the conjugate will typically possess a measurable degree of specific activity. For instance, such polymer conjugates are typically characterized as having an activity of at least about 2%, 5%, 10%, 15%, 25%, 30%, 40%, 50%, 60%, 80%, 85%, 90%, 95%, 97%, 100%, or more relative to that of the unmodified parent moiety having Factor VIII activity, when measured in a suitable model, such as those well known in the art. Preferably, compounds having a hydrolytically stable linkage (*e.g.*, an amide linkage) will possess at least some degree of the bioactivity of the unmodified parent moiety having Factor VIII activity.

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[0106] Exemplary polymer conjugates in accordance with the invention will now be described wherein the moiety having Factor VIII activity is a protein. Typically, such a protein is expected to share (at least in part) a similar amino acid sequence as native Factor VIII. Thus, while reference will be made to specific locations or atoms within the native Factor VIII protein, such a reference is for convenience only and one having ordinary skill in the art will be able to readily determine the corresponding location or atom in other moieties having Factor VIII activity. In particular, the description provided herein for native Factor VIII is applicable to Factor VIIIa, Factor VIII:vWF, and B-domain deleted Factor VIII versions, as well as fragments, deletion variants, substitution variants or addition variants of any of the foregoing.

[0107] Amino groups on Factor VIII moieties provide a point of attachment between the Factor VIII moiety and the water-soluble polymer. Native Factor VIII comprises 158 amine-containing lysine residues (6.8 weight percent of the entire protein) and one amino terminus. With respect to Factor VIIIa, there are 78 lysine residues (5.5 weight percent of the entire protein) and two amino termini (resulting from the cleavage of Factor VIII). Consequently, notwithstanding secondary and tertiary structural considerations, both Factor VIII and Factor VIIIa (as well as any peptidic Factor VIII moiety, *e.g.*, B-domain deleted Factor VIII) have several amines available for participation in conjugating reactions.

[0108] There are a number of examples of suitable water-soluble polymeric reagents useful for forming covalent linkages with available amines of a Factor VIII moiety. Specific examples, along with the corresponding conjugate, are provided in Table 1, below. In the table, the variable (n) represents the number of repeating monomeric units and "-NH-F8" represents the Factor VIII moiety following conjugation to the water-soluble polymer. While each polymeric portion presented in Table 1 terminates in a "CH₃" group, other groups (such as H and benzyl) can be substituted therefor.

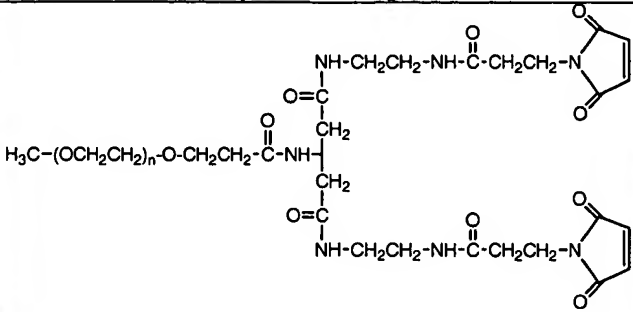
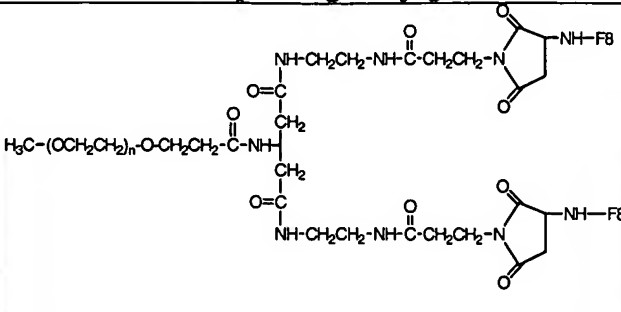
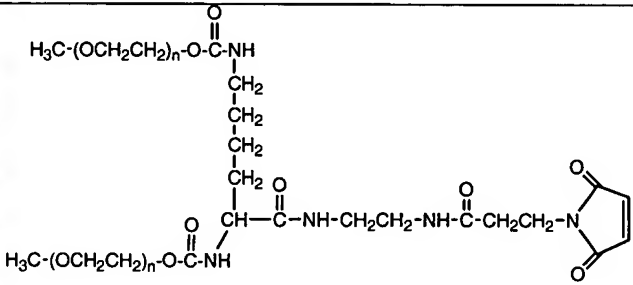
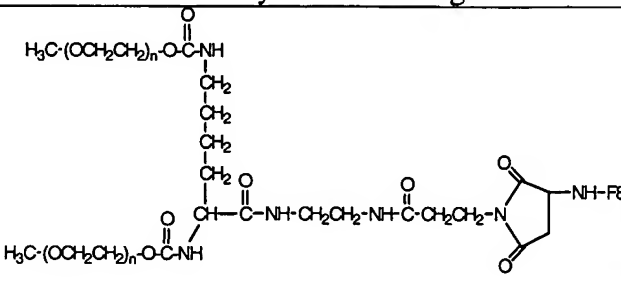
Table 1

Amine-Specific Polymeric Reagents and the Factor VIII Moiety Conjugate Formed Therefrom

Polymeric Reagent	Corresponding Conjugate
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{O}-\text{N} \begin{array}{c} \diagup \text{O} \diagdown \\ \diagdown \text{O} \diagup \end{array} \text{CH}_2$ <p>mPEG-Succinimidyl Propionate</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{NH}-\text{F8}$ <p>Amide Linkage</p>
$\begin{array}{c} \diagup \text{O} \diagdown \\ \diagdown \text{O} \diagup \end{array} \text{CH}_2-\text{N}-\text{O}-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{O}-\text{N} \begin{array}{c} \diagup \text{O} \diagdown \\ \diagdown \text{O} \diagup \end{array} \text{CH}_2$ <p>Homobifunctional PEG-Succinimidyl Propionate</p>	$\text{F8}-\text{NH}-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{NH}-\text{F8}$ <p>Amide Linkages</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{O}-\text{N} \begin{array}{c} \diagup \text{O} \diagdown \\ \diagdown \text{O} \diagup \end{array} \text{CH}_2$ <p>mPEG-Succinimidyl Butanoate</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{NH}-\text{F8}$ <p>Amide Linkage</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{C}(=\text{O})-\text{O}-\text{N} \begin{array}{c} \diagup \text{O} \diagdown \\ \diagdown \text{O} \diagup \end{array} \text{C}_6\text{H}_4$ <p>mPEG-Benzotriazole Carbonate</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{C}(=\text{O})-\text{NH}-\text{F8}$ <p>Carbamate Linkage</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{NH}-\text{C}(=\text{O})-\text{C}_6\text{H}_4-\text{O}-\text{C}(=\text{O})-\text{O}-\text{N} \begin{array}{c} \diagup \text{O} \diagdown \\ \diagdown \text{O} \diagup \end{array} \text{CH}_2$ <p>mPEG-Succinimidyl Derivative</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{NH}-\text{C}(=\text{O})-\text{C}_6\text{H}_4-\text{O}-\text{C}(=\text{O})-\text{NH}-\text{F8}$ <p>Carbamate Linkage</p>
$\begin{array}{c} \text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{C}(=\text{O})-\text{NH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2- \\ \text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{C}(=\text{O})-\text{NH}-\text{CH}-\text{C}(=\text{O})-\text{O}-\text{N} \begin{array}{c} \diagup \text{O} \diagdown \\ \diagdown \text{O} \diagup \end{array} \text{CH}_2 \end{array}$ <p>Branched mPEG2-N-Hydroxysuccinimide</p>	$\begin{array}{c} \text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{C}(=\text{O})-\text{NH}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2- \\ \text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{C}(=\text{O})-\text{NH}-\text{CH}-\text{C}(=\text{O})-\text{NH}-\text{F8} \end{array}$ <p>Amide Linkage</p>

Polymeric Reagent	Corresponding Conjugate
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{O}-\underset{\text{CH}_3}{\text{CH}}\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{O}-\text{N} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{C} \end{array}$ <p>mPEG-Succinimidyl Derivative</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{O}-\underset{\text{CH}_3}{\text{CH}}\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{F8}$ <p>Amide Linkage</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\underset{\text{CH}_3}{\text{CH}}-\overset{\text{O}}{\parallel}\text{C}-\text{O}-\text{N} \begin{array}{c} \diagup \text{O} \diagdown \\ \text{C} \end{array}$ <p>mPEG-Succinimidyl Derivative</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\underset{\text{CH}_3}{\text{CH}}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{F8}$ <p>Amide Linkage</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{S} \begin{array}{c} \diagup \text{N} \diagdown \\ \text{C} \end{array}$	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{F8}$ <p>Amide Linkage</p>
$\text{HC}=\overset{\text{O}}{\parallel}\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}$ <p>Homobifunctional PEG Propionaldehyde</p>	$\text{F8}-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{F8}$ <p>Secondary Amine Linkages</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}$ <p>mPEG Propionaldehyde</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{F8}$ <p>Secondary Amine Linkage</p>
$\text{HC}=\overset{\text{O}}{\parallel}\text{CHCH}_2\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}$ <p>Homobifunctional PEG Butyraldehyde</p>	$\text{F8}-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{F8}$ <p>Secondary Amine Linkage</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}$ <p>mPEG Butyraldehyde</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{F8}$ <p>Secondary Amine Linkage</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-(\text{CH}_2\text{CH}_2\text{O})_4-\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH}$ <p>mPEG Butyraldehyde Derivative</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-(\text{CH}_2\text{CH}_2\text{O})_4-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{F8}$ <p>Secondary Amine Linkage</p>
$\begin{array}{c} \text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2- \\ \text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-(\text{CH}_2\text{CH}_2\text{O})_4-\text{CH}_2\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{CH} \end{array}$ <p>Branched mPEG2 Butyraldehyde</p>	$\begin{array}{c} \text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2- \\ \text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-(\text{CH}_2\text{CH}_2\text{O})_4-\text{CH}_2\text{CH}_2\text{CH}_2-\text{NH}-\text{F8} \end{array}$ <p>Secondary Amine Linkage</p>

Polymeric Reagent	Corresponding Conjugate
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}(\text{OCH}_2\text{CH}_3)-\text{OCH}_2\text{CH}_3$ <p>mPEG Acetal</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{NH}-\text{F8}$ <p>Secondary Amine Linkage</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{N}(\text{C}_4\text{H}_8\text{C}_2\text{O})-\text{C}(=\text{O})$ <p>mPEG Piperidone</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{N}(\text{C}_4\text{H}_8\text{C}_2\text{O})-\text{NH}-\text{F8}$ <p>Secondary Amine Linkage (to a secondary carbon)</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-(\text{CH}_2)_{2-5}-\text{C}(=\text{O})-\text{CH}_3$ <p>mPEG Methylketone</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-(\text{CH}_2)_{2-5}-\text{CH}(\text{NH}-\text{F8})-\text{CH}_3$ <p>secondary amine linkage (to a secondary carbon)</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{N}(\text{C}_4\text{H}_3\text{O}_2)-\text{C}(=\text{O})$ <p>mPEG "Linkerless" Maleimide (under certain reaction conditions such as pH > 8)</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{N}(\text{C}_4\text{H}_3\text{O}_2)-\text{NH}-\text{F8}$ <p>Secondary Amine Linkage</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{NH}-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{N}(\text{C}_4\text{H}_3\text{O}_2)-\text{C}(=\text{O})$ <p>mPEG Maleimide Derivative (under certain reaction conditions such as pH > 8)</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{NH}-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{N}(\text{C}_4\text{H}_3\text{O}_2)-\text{NH}-\text{F8}$ <p>Secondary Amine Linkage</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{NH}-\text{CH}_2\text{CH}_2-\text{NH}-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{N}(\text{C}_4\text{H}_3\text{O}_2)-\text{C}(=\text{O})$ <p>mPEG Maleimide Derivative (under certain reaction conditions such as pH > 8)</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{C}(=\text{O})-\text{NH}-\text{CH}_2\text{CH}_2-\text{NH}-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{N}(\text{C}_4\text{H}_3\text{O}_2)-\text{NH}-\text{F8}$ <p>Secondary Amine Linkage</p>

Polymeric Reagent	Corresponding Conjugate
 <p>mPEG Forked Maleimide Derivative (under certain reaction conditions such as pH > 8)</p>	 <p>Secondary Amine Linkages</p>
 <p>branched mPEG2 Maleimide Derivative (under certain reaction conditions such as pH > 8)</p>	 <p>Secondary Amine Linkage</p>

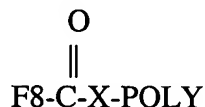
[0109] Conjugation of a polymeric reagent to an amino group of a Factor VIII moiety can be accomplished by one of ordinary skill in the art without undue experimentation. Typical of one approach is a reductive amination reaction used, for example, to conjugate a primary amine of a Factor VIII moiety with a polymer functionalized with a ketone, aldehyde or hydrated forms thereof (*e.g.*, ketone hydrate, aldehyde hydrate). In this approach, the primary amine from the Factor VIII moiety reacts with the carbonyl group of the aldehyde or ketone (or the corresponding hydroxy-containing group of a hydrated aldehyde or ketone), thereby forming a Schiff base. The Schiff base, in turn, can then be reductively converted to a stable conjugate through use of a reducing agent such as sodium borohydride. Selective reactions (*e.g.*, at the N-terminus are possible) are possible, particularly with a polymer functionalized with a ketone or an alpha-methyl branched aldehyde and/or under specific reaction conditions (*e.g.*, reduced pH).

[0110] Preferred amine groups in Factor VIII that can serve as a site for attaching a polymer include those amine groups found within the following lysine residues: Lys⁴⁹³, Lys⁴⁹⁶,

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Lys⁴⁹⁹, Lys¹⁸⁰⁴, Lys¹⁸⁰⁸, Lys¹⁸¹³, Lys¹⁸¹⁸, Lys²¹⁸³, Lys²²⁰⁷, Lys²²²⁷, Lys²²³⁶, with Lys⁴⁹⁶, Lys¹⁸⁰⁴, and Lys¹⁸⁰⁸ being particularly preferred. Numbering corresponds to the sequence provided in SEQ. ID. NO. 2. As stated above, the amine group corresponding to each of these lysine residues in a protein other than human Factor VIII can serve as a useful site for conjugation. In addition, the N-terminus of any Factor VIII moiety that is a protein can serve as a polymeric attachment site.

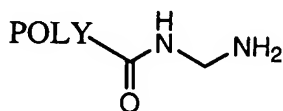
[0111] Carboxyl groups represent another functional group that can serve as a point of attachment on the Factor VIII moiety. Structurally, the conjugate will comprise the following:



where F8 and the adjacent carbonyl group corresponds to the carboxyl-containing Factor VIII moiety, X is a linkage, preferably a heteroatom selected from O, N(H), and S, and POLY is a water-soluble polymer such as PEG, optionally terminating in an end-capping moiety.

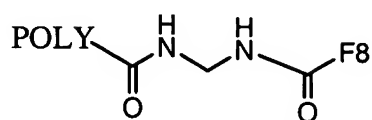
[0112] The C(O)-X linkage results from the reaction between a polymeric derivative bearing a terminal functional group and a carboxyl-containing Factor VIII moiety. As discussed above, the specific linkage will depend on the type of functional group utilized. If the polymer is end-functionalized or "activated" with a hydroxyl group, the resulting linkage will be a carboxylic acid ester and X will be O. If the polymer backbone is functionalized with a thiol group, the resulting linkage will be a thioester and X will be S. When certain multi-arm, branched or forked polymers are employed, the C(O)X moiety, and in particular the X moiety, may be relatively more complex and may include a longer linkage structure.

[0113] Water-soluble derivatives containing a hydrazide moiety are also useful for conjugation at carboxyl groups. An example of such a derivative includes a polymer having the following structure:



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which, upon conjugation with a Factor VIII moiety, has the following structure:



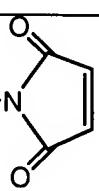
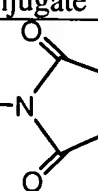
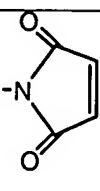
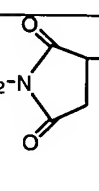
where F8 is the Factor VIII moiety following conjugation and POLY is a water-soluble polymer such as PEG, optionally terminating in an end-capping moiety.

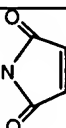
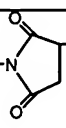
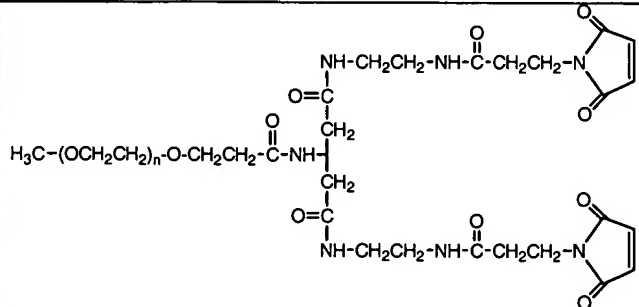
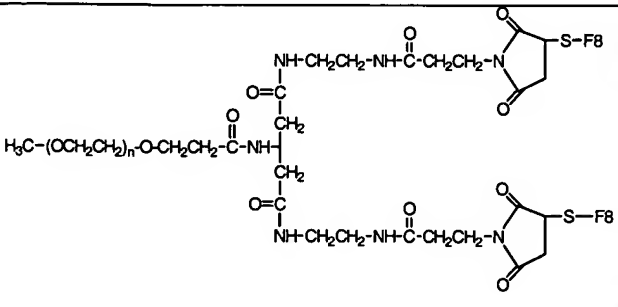
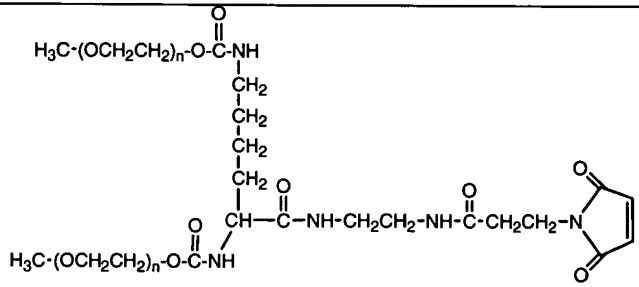
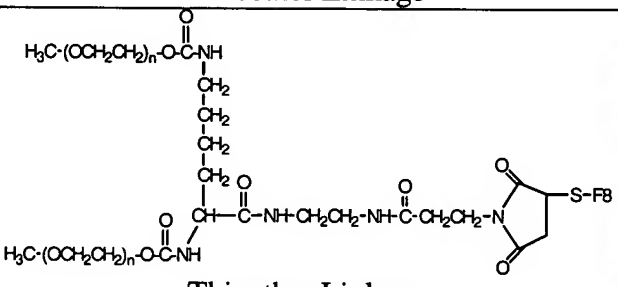
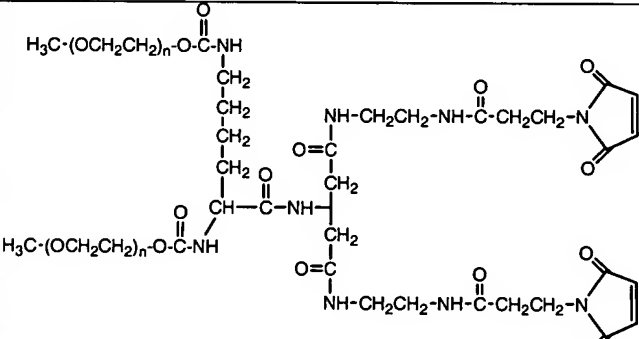
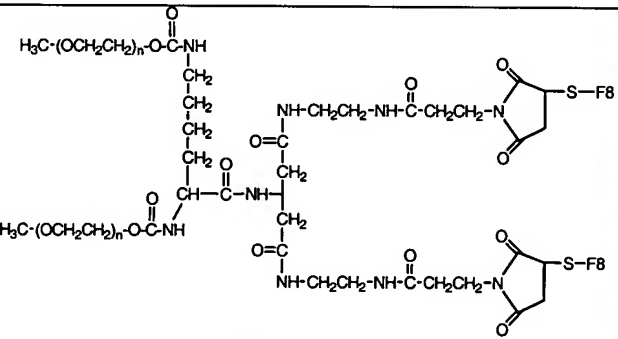
[0114] Thiol groups contained within the Factor VIII moiety can serve as effective sites of attachment for the water-soluble polymer. In particular, cysteine residues provide thiol groups when the Factor VIII moiety is a protein. The thiol groups in such cysteine residues can then be reacted with an activated PEG that is specific for reaction with thiol groups, *e.g.*, an N-maleimidyl polymer or other derivative, as described in U.S. Patent No. 5,739,208 and in International Patent Publication No. WO 01/62827.

[0115] Specific examples, along with the corresponding conjugate, are provided in Table 2, below. In the table, the variable (n) represents the number of repeating monomeric units and "-S-F8" represents the Factor VIII moiety following conjugation to the water-soluble polymer. While each polymeric portion presented in Table 1 terminates in a "CH₃" group, other groups (such as H and benzyl) can be substituted therefor.

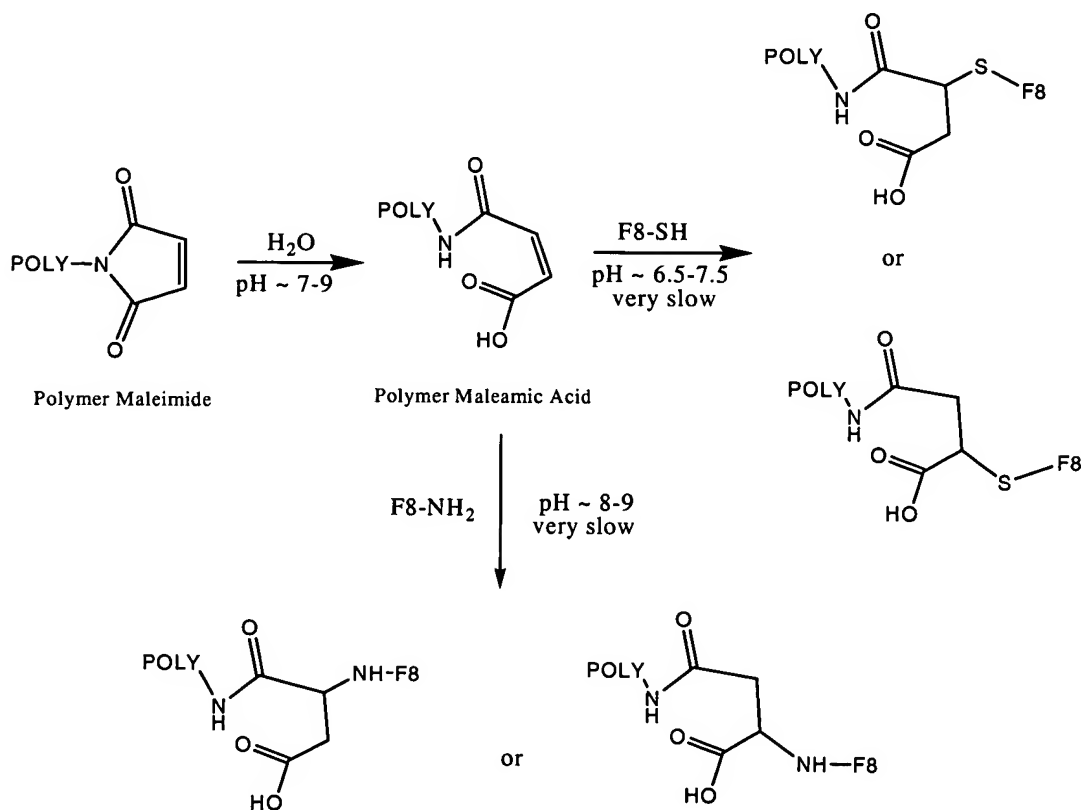
Table 2

Thiol-Specific Polymeric Reagents and the Factor VIII Moiety Conjugate Formed Therefrom

Polymeric Reagent	Corresponding Conjugate
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{N}$  mPEG "Linkerless" Maleimide	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{N}$  Thioether Linkage
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{NH}-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{N}$  mPEG Maleimide Derivative	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\text{NH}-\text{C}(=\text{O})-\text{CH}_2\text{CH}_2-\text{N}$  Thioether Linkage

Polymeric Reagent	Corresponding Conjugate
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2\text{CH}_2-\text{NH}-\overset{\text{O}}{\parallel}\text{C}-\text{CH}_2\text{CH}_2-\text{N}$  <p>mPEG Maleimide Derivative</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2\text{CH}_2-\text{NH}-\overset{\text{O}}{\parallel}\text{C}-\text{CH}_2\text{CH}_2-\text{N}$  <p>Thioether Linkage</p>
 <p>mPEG Forked Maleimide Derivative</p>	 <p>Thioether Linkage</p>
 <p>branched mPEG2 Maleimide Derivative</p>	 <p>Thioether Linkage</p>
 <p>Branched mPEG2 Forked Maleimide Derivative</p>	 <p>Thioether Linkages</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{S}-\text{CH}=\text{CH}_2$ <p>mPEG vinyl sulfone</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{S}-\text{CH}_2-\text{CH}_2-\text{S}-\text{F8}$ <p>Thioether Linkage</p>
$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{SH}$ <p>mPEG thiol</p>	$\text{H}_3\text{C}-(\text{OCH}_2\text{CH}_2)_n-\text{O}-\text{CH}_2\text{CH}_2-\overset{\text{O}}{\parallel}\text{C}-\text{NH}-\text{CH}_2-\text{CH}_2-\text{S}-\text{S}-\text{F8}$ <p>Disulfide Linkage</p>

[0116] With respect to conjugates formed from water-soluble polymers bearing one or more maleimide functional groups (regardless of whether the maleimide reacts with an amine or thiol group on the Factor VIII moiety), the corresponding maleamic acid form(s) of the water-soluble polymer can also react with the Factor VIII moiety. Under certain conditions (*e.g.*, a pH of about 7-9 and in the presence of water), the maleimide ring will "open" to form the corresponding maleamic acid. The maleamic acid, in turn, can react with an amine or thiol group of a Factor VIII moiety. Exemplary maleamic acid-based reactions are schematically shown below. POLY represents the water-soluble polymer, and F8 represents the Factor VIII moiety.



[0117] A representative conjugate in accordance with the invention can have the following structure:



wherein POLY is a water-soluble polymer, L is an optional linker, Z is a heteroatom selected from the group consisting of O, NH, and S, and Y is selected from the group consisting of C_{2-10}

alkyl, C₂₋₁₀ substituted alkyl, aryl, and substituted aryl. Polymeric reagents that can be reacted with a Factor VIII moiety and result in this type of conjugate are described in copending application filed on January 6, 2004, entitled "Thiol Selective Water Soluble Polymer Derivatives," and assigned U.S. Serial No. 10/753,047.

[0118] Preferred thiol groups in Factor VIII that can serve as a site for attaching a polymeric reagent include those thiol groups found within the following cysteine residues: Cys²⁴⁸, Cys³¹⁰, Cys³²⁹, Cys⁶³⁰, Cys⁶⁹², Cys⁷¹¹, Cys¹⁸⁹⁹, Cys¹⁹⁰³, and Cys²⁰⁰⁰, with Cys⁶³⁰, Cys⁷¹¹, and Cys¹⁹⁰³, being particularly preferred. Numbering corresponds to the sequence provided in SEQ. ID. NO. 2.

[0119] With respect to polymeric reagents, those described here and elsewhere can be purchased from commercial sources (*e.g.*, Nektar Therapeutics, Huntsville AL). In addition, methods for preparing the polymeric reagents are described in the literature.

[0120] Typically, although not necessarily, the linkage between the Factor VIII moiety and the polymeric reagent includes one or more atoms such as one or more of carbon, nitrogen, sulfur, and combinations thereof. Preferably, the linkage comprises an amide, secondary amine, carbamate, thioether, or disulfide group. Optionally, additional atoms can connect the linkage to the chain of repeating monomers within the polymeric reagent. Nonlimiting examples of specific series of atoms connecting the Factor VIII moiety to the chain of repeating monomers include those selected from the group consisting of -O-, -S-, -S-S-, -C(O)-, -O-C(O)-, -C(O)-O-, -C(O)-NH-, -NH-C(O)-NH-, -O-C(O)-NH-, -C(S)-, -CH₂-, -CH₂-CH₂-, -CH₂-CH₂-CH₂-, -CH₂-CH₂-CH₂-CH₂-, -O-CH₂-, -CH₂-O-, -O-CH₂-CH₂-, -CH₂-O-CH₂-, -CH₂-CH₂-O-, -O-CH₂-CH₂-CH₂-, -CH₂-O-CH₂-CH₂-, -CH₂-CH₂-O-CH₂-, -CH₂-CH₂-CH₂-O-, -O-CH₂-CH₂-CH₂-CH₂-, -CH₂-O-CH₂-CH₂-CH₂-, -CH₂-CH₂-O-CH₂-CH₂-, -CH₂-CH₂-O-CH₂-CH₂-, -CH₂-CH₂-CH₂-O-CH₂-, -CH₂-CH₂-CH₂-CH₂-O-, -C(O)-NH-CH₂-, -C(O)-NH-CH₂-CH₂-, -CH₂-C(O)-NH-CH₂-, -CH₂-CH₂-C(O)-NH-, -C(O)-NH-CH₂-CH₂-CH₂-, -CH₂-C(O)-NH-CH₂-CH₂-, -CH₂-CH₂-C(O)-NH-CH₂-, -CH₂-CH₂-CH₂-C(O)-NH-, -C(O)-NH-CH₂-CH₂-CH₂-CH₂-, -CH₂-C(O)-NH-CH₂-CH₂-CH₂-, -CH₂-CH₂-C(O)-NH-CH₂-CH₂-, -CH₂-CH₂-CH₂-C(O)-NH-CH₂-, -CH₂-CH₂-CH₂-CH₂-C(O)-NH-, -C(O)-O-CH₂-, -CH₂-C(O)-O-CH₂-, -CH₂-CH₂-C(O)-O-CH₂-, -C(O)-O-CH₂-CH₂-, -NH-C(O)-CH₂-,

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-CH₂-NH-C(O)-CH₂-, -CH₂-CH₂-NH-C(O)-CH₂-, -NH-C(O)-CH₂-CH₂-,
 -CH₂-NH-C(O)-CH₂-CH₂-, -CH₂-CH₂-NH-C(O)-CH₂-CH₂-, -C(O)-NH-CH₂-,
 -C(O)-NH-CH₂-CH₂-, -O-C(O)-NH-CH₂-, -O-C(O)-NH-CH₂-CH₂-,
 -O-C(O)-NH-CH₂-CH₂-CH₂-, -NH-CH₂-, -NH-CH₂-CH₂-, -CH₂-NH-CH₂-, -CH₂-CH₂-NH-CH₂-,
 -C(O)-CH₂-, -C(O)-CH₂-CH₂-, -CH₂-C(O)-CH₂-, -CH₂-CH₂-C(O)-CH₂-,
 -CH₂-CH₂-C(O)-CH₂-CH₂-, -CH₂-CH₂-C(O)-, -CH₂-CH₂-CH₂-C(O)-NH-CH₂-CH₂-NH-,
 -CH₂-CH₂-CH₂-C(O)-NH-CH₂-CH₂-NH-C(O)-,
 -CH₂-CH₂-CH₂-C(O)-NH-CH₂-CH₂-NH-C(O)-CH₂-,
 -CH₂-CH₂-CH₂-C(O)-NH-CH₂-CH₂-NH-C(O)-CH₂-CH₂-,
 -O-C(O)-NH-[CH₂]₀₋₆-(OCH₂CH₂)₀₋₂-, -C(O)-NH-(CH₂)₁₋₆-NH-C(O)-,
 -NH-C(O)-NH-(CH₂)₁₋₆-NH-C(O)-, -O-C(O)-CH₂-, -O-C(O)-CH₂-CH₂-, and
 -O-C(O)-CH₂-CH₂-CH₂-.

[0121] The conjugates are typically part of a composition. Generally, the composition comprises a plurality of conjugates, preferably although not necessarily, each having one to three water-soluble polymers covalently attached to one Factor VIII moiety. The compositions, however, can also comprise other conjugates having four, five, six, seven, eight or more polymers attached to any given moiety having Factor VIII activity. In addition, the invention includes instances wherein the composition comprises a plurality of conjugates, each conjugate comprising one water-soluble polymer covalently attached to one Factor VIII moiety, as well as compositions comprising two, three, four, five, six, seven, eight, or more water-soluble polymers covalently attached to one Factor VIII moiety.

[0122] Control of the desired number of polymers for any given moiety can be achieved by selecting the proper polymeric reagent, the ratio of polymeric reagent to the Factor VIII moiety, temperature, pH conditions, and other aspects of the conjugation reaction. In addition, reduction or elimination of the undesired conjugates (*e.g.*, those conjugates having four or more attached polymers) can be achieved through purification means.

[0123] For example, the polymer-Factor VIII moiety conjugates can be purified to obtain/isolate different conjugated species. Specifically, the product mixture can be purified to obtain an average of anywhere from one, two, three, four, five or more PEGs per Factor VIII moiety, typically one, two or three PEGs per Factor VIII moiety. The strategy for purification of

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the final conjugate reaction mixture will depend upon a number of factors, including, for example, the molecular weight of the polymeric reagent employed, the particular Factor VIII moiety, the desired dosing regimen, and the residual activity and *in vivo* properties of the individual conjugate(s).

[0124] If desired, conjugates having different molecular weights can be isolated using gel filtration chromatography and/or ion exchange chromatography. That is to say, gel filtration chromatography is used to fractionate differently numbered polymer-to-Factor VIII moiety ratios (*e.g.*, 1-mer, 2-mer, 3-mer, and so forth, wherein "1-mer" indicates 1 polymer to Factor VIII moiety, "2-mer" indicates two polymers to Factor VIII moiety, and so on) on the basis of their differing molecular weights (where the difference corresponds essentially to the average molecular weight of the water-soluble polymer portion). For example, in an exemplary reaction where a 100,000 Dalton protein is randomly conjugated to a polymeric reagent having a molecular weight of about 20,000 Daltons, the resulting reaction mixture may contain unmodified protein (having a molecular weight of about 100,000 Daltons), monoPEGylated protein (having a molecular weight of about 120,000 Daltons), diPEGylated protein (having a molecular weight of about 140,000 Daltons), and so forth.

[0125] While this approach can be used to separate PEG and other polymer-Factor VIII moiety conjugates having different molecular weights, this approach is generally ineffective for separating positional isomers having different polymer attachment sites within the Factor VIII moiety. For example, gel filtration chromatography can be used to separate from each other mixtures of PEG 1-mers, 2-mers, 3-mers, and so forth, although each of the recovered PEG-mer compositions may contain PEGs attached to different reactive amino groups (*e.g.*, lysine residues) within Factor VIII moiety.

[0126] Gel filtration columns suitable for carrying out this type of separation include Superdex™ and Sephadex™ columns available from Amersham Biosciences (Piscataway, NJ). Selection of a particular column will depend upon the desired fractionation range desired. Elution is generally carried out using a suitable buffer, such as phosphate, acetate, or the like. The collected fractions may be analyzed by a number of different methods, for example, (i) optical density (OD) at 280 nm for protein content, (ii) bovine serum albumin (BSA) protein analysis, (iii) iodine testing for PEG content (Sims *et al.* (1980) *Anal. Biochem.*, 107:60-63), and

(iv) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), followed by staining with barium iodide.

[0127] Separation of positional isomers is carried out by reverse phase chromatography using a reverse phase-high performance liquid chromatography (RP-HPLC) C18 column (Amersham Biosciences or Vydac) or by ion exchange chromatography using an ion exchange column, *e.g.*, a Sepharose™ ion exchange column available from Amersham Biosciences. Either approach can be used to separate polymer-active agent isomers having the same molecular weight (positional isomers).

[0128] The compositions are preferably substantially free of proteins that do not have Factor VIII activity. In addition, the compositions preferably are substantially free of all other noncovalently attached water-soluble polymers. Moreover, at least one species of conjugate in the composition has at least one water-soluble water polymer attached to a moiety that transforms Factor X to Factor Xa. In some circumstances, however, the composition can contain a mixture of polymer-Factor VIII moiety conjugates and unconjugated Factor VIII.

[0129] Optionally, the composition of the invention further comprises a pharmaceutically acceptable excipient. If desired, the pharmaceutically acceptable excipient can be added to a conjugate to form a composition.

[0130] Exemplary excipients include, without limitation, those selected from the group consisting of carbohydrates, inorganic salts, antimicrobial agents, antioxidants, surfactants, buffers, acids, bases, and combinations thereof.

[0131] A carbohydrate such as a sugar, a derivatized sugar such as an alditol, aldonic acid, an esterified sugar, and/or a sugar polymer may be present as an excipient. Specific carbohydrate excipients include, for example: monosaccharides, such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol, sorbitol (glucitol), pyranosyl sorbitol, myoinositol, and the like.

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[0132] The excipient can also include an inorganic salt or buffer such as citric acid, sodium chloride, potassium chloride, sodium sulfate, potassium nitrate, sodium phosphate monobasic, sodium phosphate dibasic, and combinations thereof.

[0133] The composition can also include an antimicrobial agent for preventing or deterring microbial growth. Nonlimiting examples of antimicrobial agents suitable for the present invention include benzalkonium chloride, benzethonium chloride, benzyl alcohol, cetylpyridinium chloride, chlorobutanol, phenol, phenylethyl alcohol, phenylmercuric nitrate, thimersol, and combinations thereof.

[0134] An antioxidant can be present in the composition as well. Antioxidants are used to prevent oxidation, thereby preventing the deterioration of the conjugate or other components of the preparation. Suitable antioxidants for use in the present invention include, for example, ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, hypophosphorous acid, monothioglycerol, propyl gallate, sodium bisulfite, sodium formaldehyde sulfoxylate, sodium metabisulfite, and combinations thereof.

[0135] A surfactant can be present as an excipient. Exemplary surfactants include: polysorbates, such as "Tween 20" and "Tween 80," and pluronics such as F68 and F88 (both of which are available from BASF, Mount Olive, New Jersey); sorbitan esters; lipids, such as phospholipids such as lecithin and other phosphatidylcholines, phosphatidylethanolamines (although preferably not in liposomal form), fatty acids and fatty esters; steroids, such as cholesterol; and chelating agents, such as EDTA, zinc and other such suitable cations.

[0136] Acids or bases can be present as an excipient in the composition. Nonlimiting examples of acids that can be used include those acids selected from the group consisting of hydrochloric acid, acetic acid, phosphoric acid, citric acid, malic acid, lactic acid, formic acid, trichloroacetic acid, nitric acid, perchloric acid, phosphoric acid, sulfuric acid, fumaric acid, and combinations thereof. Examples of suitable bases include, without limitation, bases selected from the group consisting of sodium hydroxide, sodium acetate, ammonium hydroxide, potassium hydroxide, ammonium acetate, potassium acetate, sodium phosphate, potassium phosphate, sodium citrate, sodium formate, sodium sulfate, potassium sulfate, potassium fumarate, and combinations thereof.

[0137] The amount of the conjugate (*i.e.*, the conjugate formed between the active agent and the polymeric reagent) in the composition will vary depending on a number of actors, but will optimally be a therapeutically effective dose when the composition is stored in a unit dose container (*e.g.*, a vial). In addition, the pharmaceutical preparation can be housed in a syringe. A therapeutically effective dose can be determined experimentally by repeated administration of increasing amounts of the conjugate in order to determine which amount produces a clinically desired endpoint.

[0138] The amount of any individual excipient in the composition will vary depending on the activity of the excipient and particular needs of the composition. Typically, the optimal amount of any individual excipient is determined through routine experimentation, *i.e.*, by preparing compositions containing varying amounts of the excipient (ranging from low to high), examining the stability and other parameters, and then determining the range at which optimal performance is attained with no significant adverse effects.

[0139] Generally, however, the excipient will be present in the composition in an amount of about 1% to about 99% by weight, preferably from about 5% to about 98% by weight, more preferably from about 15 to about 95% by weight of the excipient, with concentrations less than 30% by weight most preferred.

[0140] These foregoing pharmaceutical excipients along with other excipients are described in "Remington: The Science & Practice of Pharmacy", 19th ed., Williams & Williams, (1995), the "Physician's Desk Reference", 52nd ed., Medical Economics, Montvale, NJ (1998), and Kibbe, A.H., Handbook of Pharmaceutical Excipients, 3rd Edition, American Pharmaceutical Association, Washington, D.C., 2000.

[0141] The compositions encompass all types of formulations and in particular those that are suited for injection, *e.g.*, powders or lyophilates that can be reconstituted as well as liquids. Examples of suitable diluents for reconstituting solid compositions prior to injection include bacteriostatic water for injection, dextrose 5% in water, phosphate-buffered saline, Ringer's solution, saline, sterile water, deionized water, and combinations thereof. With respect to liquid pharmaceutical compositions, solutions and suspensions are envisioned.

[0142] The compositions of the present invention are typically, although not necessarily, administered via injection and are therefore generally liquid solutions or suspensions immediately prior to administration. The pharmaceutical preparation can also take other forms such as syrups, creams, ointments, tablets, powders, and the like. Other modes of administration are also included, such as pulmonary, rectal, transdermal, transmucosal, oral, intrathecal, subcutaneous, intra-arterial, and so forth.

[0143] The invention also provides a method for administering a conjugate as provided herein to a patient suffering from a condition that is responsive to treatment with conjugate. The method comprises administering, generally via injection, a therapeutically effective amount of the conjugate (preferably provided as part of a pharmaceutical composition). As previously described, the conjugates can be administered injected parenterally by intravenous injection, or less preferably by intramuscular or by subcutaneous injection. Suitable formulation types for parenteral administration include ready-for-injection solutions, dry powders for combination with a solvent prior to use, suspensions ready for injection, dry insoluble compositions for combination with a vehicle prior to use, and emulsions and liquid concentrates for dilution prior to administration, among others.

[0144] The method of administering may be used to treat any condition that can be remedied or prevented by administration of the conjugate. Those of ordinary skill in the art appreciate which conditions a specific conjugate can effectively treat. For example, the conjugates can be used to treat individuals suffering from hemophilia A. In addition, the conjugates are suited for use as a prophylactic against uncontrolled bleeding, optionally in patients not suffering from hemophilia. Thus, for example, the conjugate can be administered to a patient at risk for uncontrolled bleeding prior to surgery.

[0145] The actual dose to be administered will vary depend upon the age, weight, and general condition of the subject as well as the severity of the condition being treated, the judgment of the health care professional, and conjugate being administered. Therapeutically effective amounts are known to those skilled in the art and/or are described in the pertinent reference texts and literature. Generally, a therapeutically effective amount will range from about 0.001 mg to 100 mg, preferably in doses from 0.01 mg/day to 75 mg/day, and more preferably in doses from 0.10 mg/day to 50 mg/day.

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[0146] The unit dosage of any given conjugate (again, preferably provided as part of a pharmaceutical preparation) can be administered in a variety of dosing schedules depending on the judgment of the clinician, needs of the patient, and so forth. The specific dosing schedule will be known by those of ordinary skill in the art or can be determined experimentally using routine methods. Exemplary dosing schedules include, without limitation, administration five times a day, four times a day, three times a day, twice daily, once daily, three times weekly, twice weekly, once weekly, twice monthly, once monthly, and any combination thereof. Once the clinical endpoint has been achieved, dosing of the composition is halted.

[0147] One advantage of administering certain conjugates of the present invention is that individual water-soluble polymer portions can be cleaved off. Such a result is advantageous when clearance from the body is potentially a problem because of the polymer size. Optimally, cleavage of each water-soluble polymer portion is facilitated through the use of physiologically cleavable and/or enzymatically degradable linkages such as urethane, amide, carbonate or ester-containing linkages. In this way, clearance of the conjugate (via cleavage of individual water-soluble polymer portions) can be modulated by selecting the polymer molecular size and the type functional group that would provide the desired clearance properties. One of ordinary skill in the art can determine the proper molecular size of the polymer as well as the cleavable functional group. For example, one of ordinary skill in the art, using routine experimentation, can determine a proper molecular size and cleavable functional group by first preparing a variety of polymer derivatives with different polymer weights and cleavable functional groups, and then obtaining the clearance profile (e.g., through periodic blood or urine sampling) by administering the polymer derivative to a patient and taking periodic blood and/or urine sampling. Once a series of clearance profiles have been obtained for each tested conjugate, a suitable conjugate can be identified.

[0148] It is to be understood that while the invention has been described in conjunction with the preferred specific embodiments thereof, that the foregoing description as well as the examples that follow are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

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[0149] All articles, books, patents and other publications referenced herein are hereby incorporated by reference in their entireties.

EXPERIMENTAL

[0150] The practice of the invention will employ, unless otherwise indicated, conventional techniques of organic synthesis and the like, which are within the skill of the art. Such techniques are fully explained in the literature. See, for example, J. March, *Advanced Organic Chemistry: Reactions Mechanisms and Structure*, 4th Ed. (New York: Wiley-Interscience, 1992), *supra*.

[0151] In the following examples, efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.) but some experimental error and deviation should be accounted for. Unless indicated otherwise, temperature is in degrees C and pressure is at or near atmospheric pressure at sea level.

[0152] ABBREVIATIONS:

- | | | |
|--------|---------------|---|
| [0153] | DCM | dichloromethane |
| [0154] | mPEG-SPA | mPEG-succinimidyl propionate |
| [0155] | mPEG-SBA | mPEG-succinimidyl butanoate |
| [0156] | mPEG-OPSS | mPEG-orthopyridyl-disulfide |
| [0157] | mPEG-MAL | mPEG-maleimide, $\text{CH}_3\text{O}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2-\text{MAL}$ |
| [0158] | mPEG-SMB | mPEG-succinimidyl α -methylbutanoate,
$\text{CH}_3\text{O}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2-\text{CH}(\text{CH}_3)-\text{C}(\text{O})-\text{O}-\text{succinimide}$ |
| [0159] | mPEG-ButyrALD | $\text{CH}_3\text{O}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2-\text{O}-\text{C}(\text{O})-\text{NH}-(\text{CH}_2\text{CH}_2\text{O})_4\text{CH}_2\text{CH}_2\text{CH}_2\text{C}(\text{O})\text{H}$ |
| [0160] | mPEG-PIP | $\text{CH}_3\text{O}-(\text{CH}_2\text{CH}_2\text{O})_n-\text{CH}_2\text{CH}_2-\text{C}(\text{O})-\text{piperidin-4-one}$ |
| [0161] | SUC | succinimide or succinimidyl |

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[0162]	NaCNBH ₃	sodium cyanoborohydride
[0163]	HCl	hydrochloric acid
[0164]	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
[0165]	NMR	nuclear magnetic resonance
[0166]	DCC	1,3-dicyclohexylcarbodiimide
[0167]	DI	deionized
[0168]	MW	molecular weight
[0169]	r.t.	room temperature
[0170]	K or kDa	kilodaltons
[0171]	SEC	Size exclusion chromatography
[0172]	HPLC	high performance liquid chromatography
[0173]	FPLC	fast protein liquid chromatography
[0174]	SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
[0175]	MALDI-TOF	Matrix Assisted Laser Desorption Ionization Time-of-Flight
[0176]	MATERIALS:	
[0177]	All PEG reagents referred to in the appended examples are commercially available unless otherwise indicated.	
[0178]	mPEG-succinimidyl propionate, mPEG-SPA, molecular weight, 30K (M _n =31.3 kDa, Nektar Therapeutics)	
[0179]	mPEG-orthopyridyl-disulfide, mPEG-OPSS, molecular weight, 10K (M _n =10.3 kDa, Nektar Therapeutics)	
[0180]	mPEG-maleimide, mPEG-MAL, molecular weight, 20K (M _n =21.8 kDa, Nektar Therapeutics)	

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- [0181] mPEG-maleimide, mPEG-MAL, molecular weight, 30K ($M_n=31.4$ kDa, Nektar Therapeutics)
- [0182] mPEG-succinimidyl α -methylbutanoate, mPEG-SMB, molecular weight, 30K ($M_n=30.5$ kDa, Nektar Therapeutics)
- [0183] mPEG-butyraldehyde, mPEG-ButyrALD, molecular weight, 30K ($M_n=31.5$ kDa, Nektar Therapeutics)
- [0184] L-Histidine, biotechnology performance certified (Sigma)
- [0185] HEPES, biotechnology performance certified, 99.5+% (Sigma)
- [0186] Calcium chloride, dihydrate, for molecular biology, 99% (Sigma)
- [0187] Sodium chloride, for molecular biology (Sigma)
- [0188] Tween 80, Sigma Ultra, (Sigma)
- [0189] Ethyl alcohol, USP, Absolute-200 Proof (AAPER)
- [0190] Polyethylene glycol, MW 3,350, SigmaUltra (Sigma)
- [0191] Slide-A-Lyzer Dialysis Cassette, 0.5-3ml, or 3-12ml capacity (Pierce)
- [0192] Acetic acid, A.C.S. reagent, 99.7+% (Aldrich)
- [0193] 1N Acetic acid, volumetric standard (VWR)
- [0194] 1N Sodium hydroxide, volumetric standard (J.T.Baker)
- [0195] Sodium cyanoborohydride, 95% (Aldrich)
- [0196] Tris/glycine/SDS, 10x, protein electrophoresis buffer (Bio-Rad)
- [0197] Laemmli sample buffer (Bio-Rad)
- [0198] SigmaMarker, low range (M.W.6,500-66,000) (Sigma)
- [0199] SigmaMarker, high range (M.W. 36,000-205,000) (Sigma)
- [0200] 7.5% Tris-HCl ready gel (10-well, 30ul, Bio-Rad)
- [0201] GelCode blue stain reagent (Pierce)

[0202] METHODS (ANALYTICAL)**[0203]** SEC-HPLC Analysis

[0204] Size exclusion chromatography (SEC) was performed on an Agilent 1100 HPLC system (Agilent). Samples were analyzed using a BIOSEP-SEC-S 4000 column (Phenomenex) and a mobile phase of 45 mM histidine, 4.5 mM calcium chloride, 0.36 M sodium chloride, 0.009%(v/v) Tween 80 and 10% ethyl alcohol, pH 6.7. The flow rate for the column was 0.3 ml/min. Eluted protein and PEG-protein conjugates were detected by UV at a wavelength of 280nm.

[0205] SDS-PAGE Analysis

[0206] Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Mini-PROTEAN 3 Precast Gel Electrophoresis System (Bio-Rad). Samples were mixed with 2x Laemmli sample buffer, and were placed in a 95 °C water bath for ~ 5 minutes. Then, the prepared samples were loaded onto a 7.5% Tris-HCl ready gel and run for approximately 30 minutes at 200 V using Tris/glycine/SDS electrophoresis buffer.

[0207] OTHER METHODS**[0208]** Purification of PEG-Factor VIII Conjugates

[0209] A Superose 6 HR 10/30, 24 ml gel filtration column (Amersham) was used with an FPLC system and AKTA prime system (Amersham) to purify the PEG-Factor VIII conjugates in Examples 6-11. The flow rate was 0.3 ml/min and the elution buffer was 50 mM Histidine, 0.5 M NaCl, 4.0 mM CaCl₂, and 0.01% (w/v) Tween 80, pH 6.7.

[0210] Buffer exchange of Factor VIII Stock Solution

[0211] A Slide-A-Lyzer Dialysis Cassette (3-12 ml, 10,000 MWCO, Pierce) was removed from the protective pouch, and was soaked in MiliQ H₂O for 15 minutes (water was changed every 5 minutes). The Factor VIII stock solution [0.398 mg/ml in 50 mM Histidine, 0.5

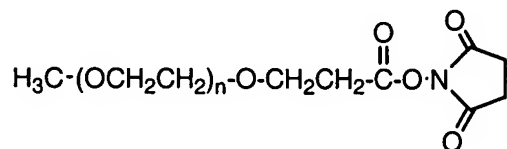
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M NaCl, 4.0 mM CaCl₂, 0.1% (w/v) PEG 3,350, 0.01% (w/v) Tween 80, pH 6.7] was then transferred into the cassette cavity through one of the guide ports on the top of the gasket. The cassette was placed in a 1 L beaker of HEPES buffer [50 mM HEPES, 0.5 M NaCl, 5 mM CaCl₂, 0.1% (w/v) PEG 3,350, 0.01% (v/v) Tween 80, pH 7.0] with a flotation buoy attached to the top of the cassette. The beaker was then placed on the stir plate to start the dialysis at 4 °C. The HEPES buffer was changed for four times at intervals of 2~3 hrs, and was then left in a cold room (4 °C) for overnight dialysis. After dialysis, the cassette chamber was injected with air and the dialyzed sample was withdrawn from the cassette. The concentration of Factor VIII in HEPES buffer was measured at UV 280nm from a SPECTRA max PLUS Spectrophotometer (Molecular Devices).

Example 1

PEGylation of B-domain Deleted Factor VIII with mPEG-SPA, 20K

[0212] mPEG-Succinimidyl propionate having a molecular weight of 20,000 Daltons is obtained from Nektar Therapeutics, (Huntsville, AL). The basic structure of the polymer reagent is provided below:



[0213] B-domain deleted Factor VIII is dissolved in deionized water, to which is added triethylamine to raise the pH to 7.2-9. To the above solution is added a 1.5 to 10-fold molar excess of the PEG reagent, mPEG-SPA. The resulting mixture is stirred at room temperature for several hours.

[0214] The reaction mixture is analyzed by SDS-PAGE to determine the degree of PEGylation of the protein. The degree of PEGylation, 1-mer, 2 mers, etc., can also be determined by any of a number of analytical techniques appropriate for proteins of this size, such as light angle scattering. The displayed peaks for native and mono-PEGylated species differ by

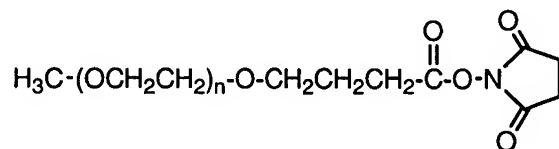
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approximately 20,000 Da. Increasing the ratio of PEG reagent to protein increases the degree of polyPEGylation, that is to say, the formation of 2-mers, 3-mers, etc.

Example 2

PEGylation of B-domain Deleted Factor VIII with mPEG-SBA

[0215] mPEG-Succinimidyl butanoate having a molecular weight of 10,000 daltons is obtained from Nektar Therapeutics, (Huntsville, AL). The basic structure of the polymer reagent is provided below:



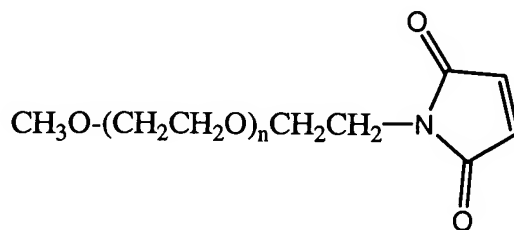
[0216] B-domain deleted Factor VIII is dissolved in deionized water, to which is added triethylamine to raise the pH to 7.2-9. To this solution is then added a 1.5 to 10-fold molar excess of mPEG-SBA. The resulting mixture is stirred at room temperature for several hours.

[0217] The reaction mixture is analyzed by SDS-PAGE to determine the degree of PEGylation of the protein.

Example 3

PEGylation of B-domain Deleted Factor VIII with mPEG-MAL, 20K

[0218] mPEG-Maleimide having a molecular weight of 20,000 daltons is obtained from Nektar Therapeutics, (Huntsville, AL). The basic structure of the polymer reagent is provided below:

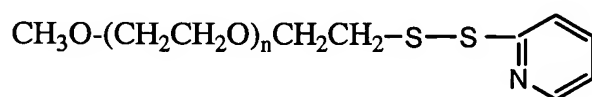


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[0219] B-domain deleted Factor VIII is dissolved in buffer. To this protein solution is added a 3-5 fold molar excess of mPEG-MAL. The mixture is stirred at room temperature under an inert atmosphere for several hours. The reaction mixture is analyzed and purified by HPLC to provide a mixture of conjugated species.

Example 4

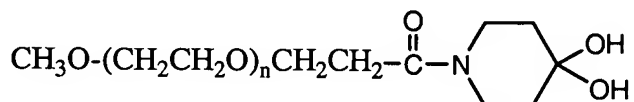
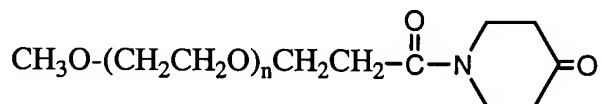
PEGylation of B-domain Deleted Factor VIII with mPEG-OPSS, 20K



[0220] The sulfhydryl-selective polymer reagent, mPEG-orthopyridyl-disulfide (structure shown above), having a molecular weight of 20,000, is obtained from Nektar Therapeutics (Huntsville, AL). A five-fold molar excess of mPEG-OPSS is added to B-domain deleted Factor VIII in a buffered solution. The reaction mixture is stirred for several hours at room temperature under an inert atmosphere to form the desired conjugate having a disulfide linkage connecting the polymer to the protein.

Example 5

PEGylation of B-domain Deleted Factor VIII with mPEG-PIP, 5K



[0221] The above polymeric reagent, shown as both the ketone and corresponding ketal, is prepared as described in Nektar Therapeutics' Provisional Patent Application No. 60/437,325, entitled "Polymer Derivatives and Conjugates Thereof."

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[0222] To prepare the above polymeric reagent, to a solution of methoxy-polyethylene glycol-succinimidyl propionate having a weight average molecular weight of 5,000 Daltons (1.0 g, 0.002 moles) in methylene chloride (20 ml), triethyl amine (0.084 ml, 0.006 moles) and 4-piperidone monohydrate hydrochloride (0.077g, 0.005 moles) are added. The reaction mixture is stirred at room temperature under a nitrogen atmosphere overnight and then purified prior to conjugation. Alternatively, the polymer reagent may be purchased from Nektar Therapeutics.

[0223] To effect conjugation, to a solution of B-domain deleted Factor VIII in aqueous buffer is added a 20-fold molar excess of mPEG-PIP, 5K. The resulting solution is placed on a Roto Mix™ orbital shaker (Thermolyne Corp., Dubuque, IA) set at slow speed to facilitate reaction at room temperature. After 15 minutes, aqueous NaCNBH₃ is added in an amount equal to a 50 fold-molar excess relative to the B-domain deleted Factor VIII. Aliquots are withdrawn at timed intervals from the reaction mixture and are analyzed by SDS-PAGE (using gels available from Bio-Rad Laboratories, Hercules, CA).

[0224] SDS-PAGE analysis indicates the presence of PEG derivatives of B-domain deleted Factor VIII having 1, 2, and 3 PEG moieties attached.

Example 6

Conjugation of B-Domain Deleted Factor VIII with mPEG-SPA, 30K

[0225] Prior to conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.

[0226] mPEG-SPA, 30K, stored at -20 °C under argon, was warmed to ambient temperature. The warmed mPEG-SPA (2.2 mg) was dissolved in 0.022 ml of 2 mM HCl to provide a 10% solution of polymer reagent. The mPEG-SPA solution was quickly added to 3 ml of Factor VIII solution [0.412mg/ml in 50 mM HEPES, 0.5 M NaCl, 4.0 mM CaCl₂, 0.1% (w/v) PEG 3,350, 0.01% (w/v) Tween 80, pH 7.0] and mixed well. After 30 minutes of reaction at room temperature, the reaction vial was transferred to a cold room (4 °C), and another 0.022 ml of mPEG-SPA solution was added to the reaction mixture, and mixed well. The pH was determined (pH 7.0±0.2). The molar ratio of mPEG-SPA to protein was 20:1. The final

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mPEG-SPA concentration was 1.445 mg/ml, and the final Factor VIII concentration was 0.406 mg/ml. The reaction was allowed to proceed overnight at 4 °C on Rotomix (slow speed, Thermolyne). The resulting conjugate was assigned identifier "pz041701."

[0227] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples.

[0228] CONJUGATE CHARACTERIZATION. The resulting conjugate mixture, prior to purification, was a mixture of Factor VII PEG-monomer (or 1-mer), dimer (or 2-mer) and trimer (or 3-mer), corresponding to the identifiers "pz041701 (low)," "pz041701 (medium)," and "pz041701 (high)" respectively, as determined by SEC. That is to say: "pz041701 (low)" corresponds to mostly Factor VIII mono-PEGylated species or Factor VIII having one PEG moiety attached thereto; "pz041701 (medium)" corresponds to primarily Factor VIII di-PEGylated species, that is to say, Factor VIII having two PEG moieties attached thereto; and "pz041701 (high)" corresponds mostly to Factor VIII having three PEG moieties attached thereto. The corresponding SEC plots are shown in FIGs. 1 and 2. FIG. 1 shows the SEC plot corresponding to fractions collected upon SEC of the Factor VIII reaction mixture. According to the size exclusion chromatography (SEC) results, the PEGylation yield of mPEG-SPA-30K-FVIII (pz041701 low) was ~ 39%. The PEGylation yield of mPEG30-SPA-30K-FVIII (pz041701 medium) was ~ 32%, and the PEGylation yield of mPEG-SPA-30K-FVIII (pz041701 high) was ~ 11%, with percentages based upon relative amounts compared to all species present in the resulting reaction mixture. The conjugate mixture was further purified by FPLC and analyzed by SDS-PAGE.

Example 7

Conjugation of B-Domain Deleted Factor VIII with mPEG-MAL, 20K

[0229] Prior to the conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.

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[0230] mPEG-MAL, 20K, stored at -20 °C under argon, was warmed to ambient temperature. The warmed mPEG-MAL reagent (4.4 mg) was dissolved in 0.044 ml of HEPES buffer [50 mM HEPES, 0.15 M NaCl, 4.0 mM CaCl₂, 0.01% (w/v) Tween 80, pH 7.0] to make a 10% mPEG-MAL solution. The mPEG-MAL solution was quickly added to 4 ml of Factor VIII solution [0.4324 mg/ml in 50 mM HEPES, 0.5 M NaCl, 4 mM CaCl₂, 0.1% (w/v) PEG 3,350, 0.01% (w/v) Tween 80, pH 7.0] and mixed well. After 30 minutes of reaction at room temperature, the reaction vial was transferred to the cold room (4 °C), and another 0.044 ml of mPEG-MAL solution was added to the reaction mixture, followed by the addition of three more aliquots of 0.044 ml of mPEG-MAL solution over the course of two hours. The pH was determined (pH 7.0±0.2). The molar ratio of mPEG-MAL to protein was 100:1. The final mPEG-MAL concentration was 5.213 mg/ml, and the final Factor VIII concentration was 0.410 mg/ml. The reaction was allowed to proceed overnight at 4 °C on Rotomix (slow speed, Thermolyne). The conjugate was assigned identifier "pz061201."

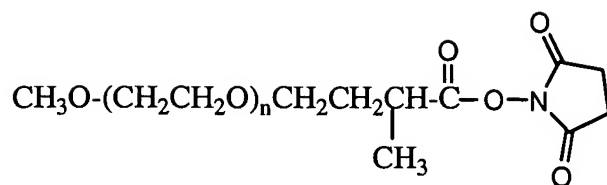
[0231] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples.

[0232] CONJUGATE CHARACTERIZATION (Mono-PEGylated product). According to the size exclusion chromatography (SEC) results, the PEGylation yield of monoPEGylated conjugate (1-mer) was ~ 33% (FIG. 3). The Factor VIII conjugate mixture fractions were combined and purified by FPLC, and then further purified by gel filtration chromatography. The pz061201 final product was analyzed by both SDS-PAGE and SEC, and the purity of the "pz061201" product was determined to be approximately 94% Factor VIII PEG monomer (*i.e.*, monopegylated Factor VIII), with ~ 6% Factor VIII PEG high-mers (FIG 4).

Example 8

Conjugation of B-Domain Deleted Factor VIII with mPEG-SMB, 30K

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[0233] Prior to conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.

[0234] mPEG-SMB, 30K, stored at $-20\text{ }^{\circ}\text{C}$ under argon, was warmed to ambient temperature. The warmed mPEG-SMB (6.5 mg) was dissolved in 0.065 ml of 2mM HCl to form a 10% mPEG-SMB solution. The mPEG-SMB solution was quickly added to 4 ml of Factor VIII solution [0.435 mg/ml in 50 mM HEPES, 0.5 M NaCl, 5.0 mM CaCl_2 , 0.1% (w/v) PEG 3,350, 0.01% (v/v) Tween 80, pH 7.0] and mixed well. After 30 minutes of reaction at room temperature, the reaction vial was transferred to a cold room ($4\text{ }^{\circ}\text{C}$). The pH was determined ($\text{pH } 7.0\pm 0.2$). The molar ratio of mPEG-SMB to protein was 20:1. The final mPEG-SMB concentration was 1.599 mg/ml, and the final Factor VIII concentration was 0.428 mg/ml. The reaction was allowed to proceed for approximately 48 hrs at $4\text{ }^{\circ}\text{C}$ on Rotomix (slow speed, Thermolyne), and was then quenched by the addition of acetic acid (99.7+%) to lower the pH to 6.0 ± 0.3 . The conjugate was assigned identifier "pz082501."

[0235] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples.

[0236] CONJUGATE CHARACTERIZATION: The mixture designated "pz082501" resulted from the PEGylation of Factor VIII with mPEG-SMB30K at $\text{pH } 7.0\pm 0.2$. The conjugate mixture was purified and analyzed by SEC. Based upon the size exclusion chromatography (SEC) results, the PEGylation yield of pz082501, monoPEGylated conjugate (Factor VIII 1-mer) was ~41% (FIG. 5). The product mixture was further purified by FPLC and analyzed by SDS-PAGE and SEC. Characterization of the purified Factor VIII PEG conjugate product, pz082501, was ~95% mono-conjugated PEG Factor VIII with ~5% higher-mers.

Example 9**Conjugation of B-Domain Deleted Factor VIII with mPEG-OPSS, 10K**

[0237] Prior to conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.

[0238] mPEG-OPSS, 10K, stored at -20 °C under argon, was warmed to ambient temperature. mPEG-OPSS (1.2 mg) was dissolved in 0.012 ml of H₂O to make a 10% mPEG-OPSS solution. The mPEG-OPSS solution was quickly added to 0.5 ml of Factor VIII solution [0.398 mg/ml in 50 mM Histidine, 0.5 M NaCl, 4.0 mM CaCl₂, 0.1% (w/v) PEG 3,350, 0.01% (w/v) Tween 80, pH 6.7] and mixed well. After 30 minutes of reaction at room temperature, the reaction vial was transferred to a cold room (4 °C). The pH was determined (pH 6.7±0.2). The molar ratio of mPEG-OPSS-10K to protein was 100:1. The final mPEG-OPSS concentration was 2.344 mg/ml, and the final Factor VIII concentration was 0.389 mg/ml. The reaction was allowed to proceed overnight at 4 °C on Rotomix (slow speed, Thermolyne).

[0239] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples. The pegylation results and yields using the mPEG-OPSS reagent were similar to those in Example 7, which employed the mPEG-MAL reagent having a molecular weight of 20K.

Example 10**Conjugation of B-Domain Deleted Factor VIII with mPEG-MAL, 30K**

[0240] Prior to conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.

[0241] mPEG-MAL, 30K, stored at -20K °C under argon, was warmed to ambient temperature. The warmed mPEG-MAL (1.0 mg) was dissolved in 0.010 ml of HEPES buffer [50 mM HEPES, 0.15 M NaCl, 4.0 mM CaCl₂, 0.01% (w/v) Tween 80, pH 7.0] to make a 10% mPEG-MAL solution. The mPEG-MAL solution was quickly added to 0.5 ml of Factor VIII solution [0.447 mg/ml in 50 mM HEPES, 0.5 M NaCl, 4 mM CaCl₂, 0.1% (w/v) PEG 3,350,

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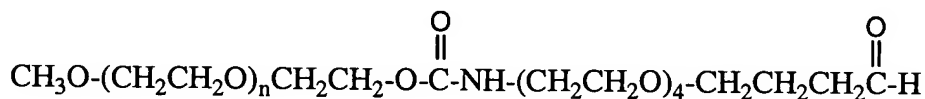
0.01% (w/v) Tween 80, pH 7.0] and mixed well. After 30 minutes of reaction at room temperature, the reaction vial was transferred to a cold room (4 °C), and another 0.010 ml of mPEG-MAL solution was added to the reaction mixture, followed by the addition of three more aliquots of 0.010 ml of mPEG-MAL solution over the course of two hours. The pH was determined (pH 7.0±0.2). The molar ratio of mPEG-MAL to protein was 100:1. The final mPEG-MAL concentration was 9.091 mg/ml, and the final Factor VIII concentration was 0.406 mg/ml. The reaction was allowed to proceed overnight at 4 °C on Rotomix (slow speed, Thermolyne).

[0242] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples. The PEGylation results and yields using the mPEG-MAL reagent having a molecular weight of 30 K were similar to those in Example 7, which employed the mPEG-MAL reagent having a molecular weight of 20K.

Example 11

Conjugation of B-Domain Deleted Factor VIII with mPEG-Butyr-ALD, 30K

[0243] Prior to conjugation, a buffer exchange for B-domain deleted Factor VIII (Factor VIII) was performed to replace histidine with HEPES.



[0244] mPEG-Butyr-ALD, 30K (shown above), stored at -20 °C under argon, was warmed to ambient temperature. The warmed mPEG-Butyr-ALD (3.8 mg) was dissolved in 0.038 ml of H₂O to make a 10% mPEG-Butyr-ALD solution. The mPEG-Butyr-ALD solution was quickly added to 0.5 ml of Factor VIII solution [0.400 mg/ml in 50 mM HEPES, 0.5 M NaCl, 5 mM CaCl₂, 0.1% (w/v) PEG 3,350, 0.01% (v/v) Tween 80, pH 7.0] and mixed well. After 15 minutes, 0.060 ml of 10 mM sodium cyanoborohydride solution was added. The pH

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was determined (pH 7.0 ± 0.2). The molar ratio of mPEG-Butyr-ALD to protein was 100:1. The final mPEG-Butyr-ALD concentration was 6.355 mg/ml. The final Factor VIII concentration was 0.334 mg/ml, and the final concentration of NaCNBH₃ was 1.003 mM. The reaction was allowed to proceed for 5 hours at room temperature, and then, overnight at 4 °C on Rotomix (slow speed, Thermolyne).

[0245] The conjugate mixture was purified using gel filtration chromatography. A size exclusion chromatography method was developed for analyzing the reaction mixtures, and the final products. SDS-PAGE analysis was also used for the characterization of the samples. The yield of Factor VIII mono-PEG conjugate was approximately 20%.

Example 12

In-vitro Activity of Exemplary Factor VIII-PEG Conjugates

[0246] The in-vitro activities of the Factor VIII-PEG conjugates described in Examples 6, 7, and 8 were determined. All of the Factor VIII conjugates tested were bioactive.

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SEQUENCE LISTING

<110> Bentley, Michael
Mary J. Bossard

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<140> To Be Assigned

<141> 2003-02-26

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Pro	Ser	Lys	Ala	Arg	Leu	His	Leu	Gln	Gly	Arg	Ser	Asn	Ala	Trp
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Arg	Pro	Gln	Val	Asn	Asn	Pro	Lys	Glu	Trp	Leu	Gln	Val	Asp	Phe
2240						2245					2250			
Gln	Lys	Thr	Met	Lys	Val	Thr	Gly	Val	Thr	Thr	Gln	Gly	Val	Lys
2255						2260					2265			
Ser	Leu	Leu	Thr	Ser	Met	Tyr	Val	Lys	Glu	Phe	Leu	Ile	Ser	Ser
2270						2275					2280			
Ser	Gln	Asp	Gly	His	Gln	Trp	Thr	Leu	Phe	Phe	Gln	Asn	Gly	Lys
2285						2290					2295			
Val	Lys	Val	Phe	Gln	Gly	Asn	Gln	Asp	Ser	Phe	Thr	Pro	Val	Val
2300						2305					2310			
Asn	Ser	Leu	Asp	Pro	Pro	Leu	Leu	Thr	Arg	Tyr	Leu	Arg	Ile	His
2315						2320					2325			
Pro	Gln	Ser	Trp	Val	His	Gln	Ile	Ala	Leu	Arg	Met	Glu	Val	Leu
2330						2335					2340			

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Gly Cys Glu Ala Gln Asp Leu Tyr
 2345 2350

<210> 2
 <211> 2332
 <212> PRT
 <213> Homo sapiens

<400> 2

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
 1 5 10 15
 Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
 20 25 30
 Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
 35 40 45
 Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
 50 55 60
 Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
 65 70 75 80
 Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
 85 90 95
 Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
 100 105 110
 Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
 115 120 125
 Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
 130 135 140
 Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
 145 150 155 160
 His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
 165 170 175
 Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
 180 185 190
 His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
 195 200 205
 His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
 210 215 220
 Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
 225 230 235 240
 Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
 245 250 255
 Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
 260 265 270

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Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
 595 600 605
 Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
 610 615 620
 Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
 625 630 635 640
 Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
 645 650 655
 Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
 660 665 670
 Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
 675 680 685
 Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
 690 695 700
 Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
 705 710 715 720
 Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
 725 730 735
 Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro Ser Thr Arg
 740 745 750
 Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
 755 760 765
 Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
 770 775 780
 Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
 785 790 795 800
 His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
 805 810 815
 Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
 820 825 830
 Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
 835 840 845
 Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
 850 855 860
 Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser
 865 870 875 880
 Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala
 885 890 895
 Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
 900 905 910

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Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
 915 920 925
 Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
 930 935 940
 Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp
 945 950 955 960
 Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
 965 970 975
 Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
 980 985 990
 Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala
 995 1000 1005
 Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu
 1010 1015 1020
 Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu
 1025 1030 1035
 Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp
 1040 1045 1050
 Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr
 1055 1060 1065
 Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly
 1070 1075 1080
 Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met Ser Phe Phe Lys
 1085 1090 1095
 Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile Gln Arg Thr His
 1100 1105 1110
 Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro Ser Pro Lys Gln
 1115 1120 1125
 Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu Gly Gln Asn Phe
 1130 1135 1140
 Leu Ser Glu Lys Asn Lys Val Val Val Gly Lys Gly Glu Phe Thr
 1145 1150 1155
 Lys Asp Val Gly Leu Lys Glu Met Val Phe Pro Ser Ser Arg Asn
 1160 1165 1170
 Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu Asn Asn Thr His
 1175 1180 1185
 Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu Lys Lys Glu Thr
 1190 1195 1200
 Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr Val Thr
 1205 1210 1215

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Gly Thr	Lys Asn Phe Met	Lys	Asn Leu Phe Leu	Leu	Ser Thr Arg
1220		1225		1230	
Gln Asn	Val Glu Gly Ser	Tyr	Asp Gly Ala Tyr	Ala	Pro Val Leu
1235		1240		1245	
Gln Asp	Phe Arg Ser Leu	Asn	Asp Ser Thr Asn	Arg	Thr Lys Lys
1250		1255		1260	
His Thr	Ala His Phe Ser	Lys	Lys Gly Glu Glu	Glu	Asn Leu Glu
1265		1270		1275	
Gly Leu	Gly Asn Gln Thr	Lys	Gln Ile Val Glu	Lys	Tyr Ala Cys
1280		1285		1290	
Thr Thr	Arg Ile Ser Pro	Asn	Thr Ser Gln Gln	Asn	Phe Val Thr
1295		1300		1305	
Gln Arg	Ser Lys Arg Ala	Leu	Lys Gln Phe Arg	Leu	Pro Leu Glu
1310		1315		1320	
Glu Thr	Glu Leu Glu Lys	Arg	Ile Ile Val Asp	Asp	Thr Ser Thr
1325		1330		1335	
Gln Trp	Ser Lys Asn Met	Lys	His Leu Thr Pro	Ser	Thr Leu Thr
1340		1345		1350	
Gln Ile	Asp Tyr Asn Glu	Lys	Glu Lys Gly Ala	Ile	Thr Gln Ser
1355		1360		1365	
Pro Leu	Ser Asp Cys Leu	Thr	Arg Ser His Ser	Ile	Pro Gln Ala
1370		1375		1380	
Asn Arg	Ser Pro Leu Pro	Ile	Ala Lys Val Ser	Ser	Phe Pro Ser
1385		1390		1395	
Ile Arg	Pro Ile Tyr Leu	Thr	Arg Val Leu Phe	Gln	Asp Asn Ser
1400		1405		1410	
Ser His	Leu Pro Ala Ala	Ser	Tyr Arg Lys Lys	Asp	Ser Gly Val
1415		1420		1425	
Gln Glu	Ser Ser His Phe	Leu	Gln Gly Ala Lys	Lys	Asn Asn Leu
1430		1435		1440	
Ser Leu	Ala Ile Leu Thr	Leu	Glu Met Thr Gly	Asp	Gln Arg Glu
1445		1450		1455	
Val Gly	Ser Leu Gly Thr	Ser	Ala Thr Asn Ser	Val	Thr Tyr Lys
1460		1465		1470	
Lys Val	Glu Asn Thr Val	Leu	Pro Lys Pro Asp	Leu	Pro Lys Thr
1475		1480		1485	
Ser Gly	Lys Val Glu Leu	Leu	Pro Lys Val His	Ile	Tyr Gln Lys
1490		1495		1500	
Asp Leu	Phe Pro Thr Glu	Thr	Ser Asn Gly Ser	Pro	Gly His Leu
1505		1510		1515	

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Asp	Leu	Val	Glu	Gly	Ser	Leu	Leu	Gln	Gly	Thr	Glu	Gly	Ala	Ile
1520						1525					1530			
Lys	Trp	Asn	Glu	Ala	Asn	Arg	Pro	Gly	Lys	Val	Pro	Phe	Leu	Arg
1535						1540					1545			
Val	Ala	Thr	Glu	Ser	Ser	Ala	Lys	Thr	Pro	Ser	Lys	Leu	Leu	Asp
1550						1555					1560			
Pro	Leu	Ala	Trp	Asp	Asn	His	Tyr	Gly	Thr	Gln	Ile	Pro	Lys	Glu
1565						1570					1575			
Glu	Trp	Lys	Ser	Gln	Glu	Lys	Ser	Pro	Glu	Lys	Thr	Ala	Phe	Lys
1580						1585					1590			
Lys	Lys	Asp	Thr	Ile	Leu	Ser	Leu	Asn	Ala	Cys	Glu	Ser	Asn	His
1595						1600					1605			
Ala	Ile	Ala	Ala	Ile	Asn	Glu	Gly	Gln	Asn	Lys	Pro	Glu	Ile	Glu
1610						1615					1620			
Val	Thr	Trp	Ala	Lys	Gln	Gly	Arg	Thr	Glu	Arg	Leu	Cys	Ser	Gln
1625						1630					1635			
Asn	Pro	Pro	Val	Leu	Lys	Arg	His	Gln	Arg	Glu	Ile	Thr	Arg	Thr
1640						1645					1650			
Thr	Leu	Gln	Ser	Asp	Gln	Glu	Glu	Ile	Asp	Tyr	Asp	Asp	Thr	Ile
1655						1660					1665			
Ser	Val	Glu	Met	Lys	Lys	Glu	Asp	Phe	Asp	Ile	Tyr	Asp	Glu	Asp
1670						1675					1680			
Glu	Asn	Gln	Ser	Pro	Arg	Ser	Phe	Gln	Lys	Lys	Thr	Arg	His	Tyr
1685						1690					1695			
Phe	Ile	Ala	Ala	Val	Glu	Arg	Leu	Trp	Asp	Tyr	Gly	Met	Ser	Ser
1700						1705					1710			
Ser	Pro	His	Val	Leu	Arg	Asn	Arg	Ala	Gln	Ser	Gly	Ser	Val	Pro
1715						1720					1725			
Gln	Phe	Lys	Lys	Val	Val	Phe	Gln	Glu	Phe	Thr	Asp	Gly	Ser	Phe
1730						1735					1740			
Thr	Gln	Pro	Leu	Tyr	Arg	Gly	Glu	Leu	Asn	Glu	His	Leu	Gly	Leu
1745						1750					1755			
Leu	Gly	Pro	Tyr	Ile	Arg	Ala	Glu	Val	Glu	Asp	Asn	Ile	Met	Val
1760						1765					1770			
Thr	Phe	Arg	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Ser	Phe	Tyr	Ser	Ser
1775						1780					1785			
Leu	Ile	Ser	Tyr	Glu	Glu	Asp	Gln	Arg	Gln	Gly	Ala	Glu	Pro	Arg
1790						1795					1800			
Lys	Asn	Phe	Val	Lys	Pro	Asn	Glu	Thr	Lys	Thr	Tyr	Phe	Trp	Lys
1805						1810					1815			

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Val	Gln	His	His	Met	Ala	Pro	Thr	Lys	Asp	Glu	Phe	Asp	Cys	Lys
1820						1825					1830			
Ala	Trp	Ala	Tyr	Phe	Ser	Asp	Val	Asp	Leu	Glu	Lys	Asp	Val	His
1835						1840					1845			
Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Val	Cys	His	Thr	Asn	Thr	Leu
1850						1855					1860			
Asn	Pro	Ala	His	Gly	Arg	Gln	Val	Thr	Val	Gln	Glu	Phe	Ala	Leu
1865						1870					1875			
Phe	Phe	Thr	Ile	Phe	Asp	Glu	Thr	Lys	Ser	Trp	Tyr	Phe	Thr	Glu
1880						1885					1890			
Asn	Met	Glu	Arg	Asn	Cys	Arg	Ala	Pro	Cys	Asn	Ile	Gln	Met	Glu
1895						1900					1905			
Asp	Pro	Thr	Phe	Lys	Glu	Asn	Tyr	Arg	Phe	His	Ala	Ile	Asn	Gly
1910						1915					1920			
Tyr	Ile	Met	Asp	Thr	Leu	Pro	Gly	Leu	Val	Met	Ala	Gln	Asp	Gln
1925						1930					1935			
Arg	Ile	Arg	Trp	Tyr	Leu	Leu	Ser	Met	Gly	Ser	Asn	Glu	Asn	Ile
1940						1945					1950			
His	Ser	Ile	His	Phe	Ser	Gly	His	Val	Phe	Thr	Val	Arg	Lys	Lys
1955						1960					1965			
Glu	Glu	Tyr	Lys	Met	Ala	Leu	Tyr	Asn	Leu	Tyr	Pro	Gly	Val	Phe
1970						1975					1980			
Glu	Thr	Val	Glu	Met	Leu	Pro	Ser	Lys	Ala	Gly	Ile	Trp	Arg	Val
1985						1990					1995			
Glu	Cys	Leu	Ile	Gly	Glu	His	Leu	His	Ala	Gly	Met	Ser	Thr	Leu
2000						2005					2010			
Phe	Leu	Val	Tyr	Ser	Asn	Lys	Cys	Gln	Thr	Pro	Leu	Gly	Met	Ala
2015						2020					2025			
Ser	Gly	His	Ile	Arg	Asp	Phe	Gln	Ile	Thr	Ala	Ser	Gly	Gln	Tyr
2030						2035					2040			
Gly	Gln	Trp	Ala	Pro	Lys	Leu	Ala	Arg	Leu	His	Tyr	Ser	Gly	Ser
2045						2050					2055			
Ile	Asn	Ala	Trp	Ser	Thr	Lys	Glu	Pro	Phe	Ser	Trp	Ile	Lys	Val
2060						2065					2070			
Asp	Leu	Leu	Ala	Pro	Met	Ile	Ile	His	Gly	Ile	Lys	Thr	Gln	Gly
2075						2080					2085			
Ala	Arg	Gln	Lys	Phe	Ser	Ser	Leu	Tyr	Ile	Ser	Gln	Phe	Ile	Ile
2090						2095					2100			
Met	Tyr	Ser	Leu	Asp	Gly	Lys	Lys	Trp	Gln	Thr	Tyr	Arg	Gly	Asn
2105						2110					2115			

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Ser Thr Gly Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser	2120	2125	2130
Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr	2135	2140	2145
Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser Thr Leu Arg	2150	2155	2160
Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys Ser Met Pro Leu	2165	2170	2175
Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser	2180	2185	2190
Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser Pro Ser Lys Ala	2195	2200	2205
Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp Arg Pro Gln Val	2210	2215	2220
Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe Gln Lys Thr Met	2225	2230	2235
Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys Ser Leu Leu Thr	2240	2245	2250
Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser Ser Gln Asp Gly	2255	2260	2265
His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys Val Lys Val Phe	2270	2275	2280
Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn Ser Leu Asp	2285	2290	2295
Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp	2300	2305	2310
Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly Cys Glu Ala	2315	2320	2325
Gln Asp Leu Tyr	2330		